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FACULTY OF SCIENCES

# Screening of the Vascular Endothelial Growth Factor family in human colon cancer

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Promoter: Prof. Dr. Johan Grooten

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# List of abbreviations

| | | |
|---|---|---|
| | 5-FU | 5-Fluorouracil |
| **A** | Akt | v-akt Murine thymoma viral oncogene |
| | Ang | Angiopoietin |
| | AP-1/2 | Activator protein 1/2 |
| | APC | Adenomatous polyposis coli |
| | ApoE | Apolipoprotein E |
| | AUC | Area under the curve |
| **B** | Bcl-2/$X_L$ | B-cell lymphoma 2/extra-large |
| | bFGF | basic Fibroblast growth factor |
| | BLT | Leukotriene $B_4$ receptor |
| | BM | Bone marrow |
| | BMDC | Bone marrow-derived cell |
| | BRAF | v-raf Murine sarcoma viral oncogene homolog B1 |
| **C** | CAIX | Carbonic anhydrase IX |
| | cDNA | complementary DNA |
| | c-Fms | Colony stimulating factor 1 receptor |
| | c-Kit | v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog |
| | c-Met | Hepatocyte growth factor receptor |
| | c-myc | v-myc Myelocytomatosis viral oncogene homolog |
| | CEA | Carcinoembryonic antigen |
| | COX | Cyclooxygenase |
| | COXIB | Cyclooxygenase inhibitor |
| | CREB | cAMP-Responsive element-binding protein |
| | CSF | Colony stimulating factor |
| | CSF-1R | Colony Stimulating Factor 1 Receptor |
| | CT | X-Ray computed tomography |
| | CXCL | C-X-C motif ligand |
| | CysLT | Cysteinyl leukotriene receptor |
| **D** | DCC | Deleted in colorectal cancer |
| | DMSO | Dimethylsulfoxide |
| | DNA | Deoxyribonucleic acid |
| **E** | ECM | Extracellular matrix |

| | | |
|---|---|---|
| | EETs | Epoxyeicosatrienoic acids |
| | EGF | Epidermal growth factor |
| | EGFR | Epidermal growth factor receptor |
| | Egr-1 | Early growth response factor-1 |
| | EMEA | European Medicines Agency |
| | eNOS | Endothelial nitric oxide synthase |
| | EP1-4 | Prostaglandin E receptor 1-4 |
| | EPA | Eicosapentaenoic acid |
| | ERK | Extracellular signal-regulated kinase |
| **F** | FAP | Familial adenomatous polyposis |
| | FDA | Food and Drug Administration |
| | FGF | Fibroblast growth factor |
| | FGFR | Fibroblast growth factor receptor |
| | FIGF | c-Fos induced growth factor |
| | FLAP | 5-Lipoxygease activating protein |
| | Flk-1 | Fetal liver kinase-1 |
| | Flt-1/4 | Fms-like tyrosine kinase-1/4 |
| | FOLFOX | 5-FU, leucovorin and oxaliplatin |
| **G** | G-CSF | Granulocyte colony-stimulating factor |
| | gDNA | genomic DNA |
| | GLUT-1 | Glucose transporter 1 |
| | γGT | γ-Glutamyl transferase |
| | GTP | Guanosine triphosphate |
| **H** | HER2 | Human epidermal growth factor receptor 2 |
| | HETEs | Hydroxyeicosatetraenoic acids |
| | HIF | Hypoxia inducible factor |
| | HNPCC | Hereditary non-polyposis colorectal cancer |
| | HODE | Hydroxyoctadecadienoic acid |
| | HPETEs | Hydroperoxyeicosatetraenoic acids |
| | HRE | Hypoxia responsive element |
| **I** | IAP-1/2 | Inhibitor of apoptosis protein |
| | Ig | Immunoglobulin |
| | IFL | Irinotecan, 5-FU and leucovorin |
| | IL | Interleukin |
| | INFγ | Interferon-γ |
| | iNOS | Inducible nitric oxide synthase |
| | Itk | Interleukin-2 receptor inducible T-cell kinase |
| **J** | JNK | JUN N-terminal kinase |

| | | |
|---|---|---|
| **K** | KDR | Kinase domain-containing receptor |
| | KRAS | Kirsten rat sarcoma viral oncogene homolog |
| **L** | Lck | Leukocyte-specific protein tyrosine kinase |
| | LOX | Lipoxygenase |
| | LT | Leukotriene |
| | LVEC | Lymphatic vessel endothelial cell |
| | LXRalpha | Liver X receptor alpha |
| | LYVE | Lymphatic vessel endothelial hyaluronan receptor |
| **M** | MCP | Monocyte chemoattractant protein |
| | MLH | Mutation of human mutL homolog |
| | MMP | Matrix metalloproteinase |
| | MRI | Magnetic resonance imaging |
| | mRNA | messenger Ribonucleic acid |
| | MSH | Human mutS homolog |
| | MTF1 | Metal Transcription Factor 1 |
| **N** | NCCN | National comprehensive cancer network |
| | NF-κB | Nuclear factor-kappa B |
| | NP | Neuropilin |
| | NSAIDS | Non-steroidal anti-inflammatory drugs |
| **P** | P53 | 53kDa tumor protein |
| | PCR | Polymerase chain reaction |
| | PDGF | Platelet-derived growth factor |
| | PDGFR | Platelet-derived growth factor receptor |
| | PG | Prostaglandin |
| | PI3K | Phosphatidylinositol 3 kinase |
| | PKA | Protein kinase A/ cAMP-dependent protein kinase |
| | PKC | Protein kinase C |
| | PlGF | Placental growth factor |
| | PPARδ | Peroxisome proliferator-activated receptor-delta |
| | Prox1 | Prospero homeobox protein 1 |
| | Ptges | Prostaglandin E synthase |
| | PUFA | Polyunsaturated fatty acid |
| **R** | Rac | Ras-related C3 botulinum toxin substrate |
| | Raf | Murine leukemia viral oncogenehomolog |
| | Ras | Rat sarcoma viral oncogene homolog |
| | RET | Ret proto-oncogene |

| | | |
|---|---|---|
| | ROC | Receiver operator characteristic |
| | RT-qPCR | Real-time quantitative PCR |
| **S** | SDF-1 | Stromal cell-derived factor-1 |
| | SDHA | Succinate dehydrogenase complex subunit A |
| | Src | v-src Sarcoma viral oncogene homolog |
| | Sp1 | Specificity protein 1 |
| | STAT3 | Signal transducer activator of transcription 3 |
| **T** | TBP | TATA-binding protein |
| | TGF | Transforming growth factor |
| | TFF3 | Trefoil factor 3 |
| | TFPI-2 | Tissue factor pathway inhibitor-2 |
| | TLR | Toll like receptor |
| | TME | Total mesorectal excision |
| | TNF | Tumor necrosis factor |
| | TNM | Tumor, node, metastases |
| | TWIST | Twist basic helix-loop-helix transcription factor |
| | $TXA_2$ | Tromboxane $A_2$ |
| **U** | uPAR | urokinase Plasminogen activator receptor |
| | USPSTF | United States Preventive Services Task Force |
| **V** | VEGF | Vascular endothelial growth factor |
| | VEGFR | Vascular endothelial growth factor receptor |
| | VEGFs | VEGF family members |
| **X** | XIAP | X-linked inhibitor of apoptosis protein |

# Summary

VEGF family members play a pivotal role in angiogenesis and lymphangiogenesis, two important biological responses that crucially contribute to the outgrowth of solid tumors and the development of metastases. Despite the importance of VEGF-A in neo-angiogenesis, drugs interfering with VEGF-A only show modest effects in most cancers and resistance tends to develop after a transitory period of clinical benefit, resulting in regrowth of the tumors and progression of the disease. Angiogenic escape involving the compensatory expression of VEGF-related and non-related angiogenic factors has been proposed to contribute to this limited response to VEGF-A directed angiogenic therapy. Within this context, we performed an expression profiling of the human VEGF family members (VEGF-A, VEGF-B, VEGF-C, VEGF-D and PlGF) in different stages of colon cancer, namely colon adenoma, carcinoma and liver metastasis. A literature search for the expression of these angiogenic genes yields contradictory results due to methodological differences and fragmented approaches of studies on VEGFs expression in colon cancer. Therefore, we performed a systematic and comprehensive expression analysis, determining in a single experimental setup the mRNA expression levels of all human VEGF family members during the progression from colon adenoma to carcinoma and liver metastasis. A preliminary study revealed that colon samples obtained by surgical resection are susceptible to hypoxia elicited during the surgical procedure. This sampling-induced hypoxia in resection samples but not in biopsy samples affects the marker-reliability of VEGF family members. Therefore, biopsy samples provide a more accurate report on VEGF family mRNA levels.

The results obtained by our expression analysis provide important new insights into the redundant expression of VEGF family members in colon carcinoma and their association with progression from adenoma to carcinoma to metastasis. In addition to *VEGF-A,* especially *PlGF* emerges from our analysis as a prominently expressed VEGF member in all stages of colon cancer. *VEGF-B* and *VEGF*-C were identified as angiogenic genes upregulated only in carcinoma and metastasis samples. Analysis of individual patient expression signatures revealed major progression-associated shifts in the VEGF signatures. Whereas colon adenoma patients showed overexpression of one or two VEGFs, a shift toward the expression of multiple VEGF family

members was observed with disease progression. Hence, patients with colon carcinoma or liver metastases had a predominant overexpression of three or four VEGF family members. In view of this multiplicity of *VEGF* expression, we found a remarkable conservation of angiogenic signatures between colon carcinomas and liver metastases. The striking broadening of angiogenic gene expression with tumor progression in samples from untreated patients provides an explanatory basis for the differing therapeutic outcomes of targeting a single VEGF factor such as VEGF-A as well as for the overall weak to modest efficacy of anti-angiogenic therapy.

Considering eicosanoids as potential drivers for the VEGFs expression signatures, a correlated analysis of *COX2*, *5-LOX* and *VEGFs* mRNA expression revealed the complex and intertwined nature of inflammatory and angiogenic gene expression already at the stage of adenoma. Distinctive *5-LOX* and *COX2* expression profiles were revealed for colon adenoma and carcinoma and the presence of these enzymes was clearly associated with cumulative co-expression of VEGF family members. Finally, in colon adenoma, the dual expression of *COX2* and *5-LOX* may indicate an increased risk for malignant transformation. Considering the observed association between eicosanoids and co-expression of VEGF family members, colon cancer patients may benefit from a combination therapy targeting angiogenic VEGF signaling as well as eicosanoids to additionally suppress the co-expression of multiple VEGFs.

# Samenvatting

De VEGF familieleden spelen een vooraanstaande rol in angiogenese en lymfangiogenese, twee belangrijke biologische processen die een cruciale bijdrage leveren aan de uitgroei van vaste tumoren en de ontwikkeling van metastasen. Therapie gericht tegen de angiogene sleutelfactor VEGF-A heeft echter niet het verwachte effect en na verloop van tijd hervallen de meeste patiënten. Angiogene ontsnappingsmechanismen waarbij andere factoren de rol van VEGF-A gaan uitvoeren, liggen waarschijnlijk aan de beperkte werking van therapie gericht tegen VEGF-A. In deze context hebben we een expressieprofilering van de verschillende VEGF familieleden (VEGF-A, VEGF-B, VEGF-C, VEGF-D en PlGF) uitgevoerd in verschillende stadia van colonkanker, namelijk colonadenoma, coloncarcinoma en levermetastasen. Een literatuurstudie naar de expressie van deze angiogene genen levert tegenstrijdige resultaten door de verschillende methodologische en gefragmenteerde benaderingen van studies naar de expressie van VEGFs in colonkanker. Daarom hebben wij een systematische en uitvoerige expressie-analyse uitgevoerd om in een enkele experimentele opstelling de mRNA expressie levels van alle VEGF familieleden te bepalen tijdens de progressie van colonadenoma naar -carcinoma en naar levermetastasen. Een voorafgaand onderzoek onthulde dat colonstalen verkregen via chirurgische resectie onderhevig zijn aan hypoxie die veroorzaakt wordt tijdens de chirurgische procedure. Deze hypoxie geïnduceerd door de staalname heeft in resectiestalen een invloed op de betrouwbaarheid van de VEGF familieleden als merker voor colonkanker. Daarom leveren stalen verkregen via biopsie een meer accuraat zicht op de mRNA expressie van de VEGF familieleden.

De resultaten die bekomen werden in onze expressie-analyse leveren belangrijke nieuwe inzichten over de redundante expressie van VEGF familieleden in colonkanker en hun associatie met progressie van adenoma naar carcinoma en metastasen. Naast *VEGF-A*, blijkt vooral *PlGF* uit onze analyse naar voor te komen als een VEGF-lid met een prominente expressie in alle stadia van colonkanker. *VEGF-B* en *VEGF-C* werden geïdentificeerd als tumorspecifieke angiogene genen met enkel een opregulering in stalen van carcinoma en metastasen. Analyse van de expressie-signaturen van individuele patiënten onthulde aanzienlijke verschuivingen die geassocieerd waren met progressie. Waar colonadenoma patiënten een overexpressie van een of twee VEGFs toonden, was een verschuiving naar de expressie van meerdere VEGF familieleden

waarneembaar bij progressie van de ziekte. Zo hadden patiënten met colonkanker of levermetastasen een uitgesproken overexpressie van drie of vier VEGF familieleden. Tevens vonden we een opmerkelijke bewaring van de angiogene signaturen tussen stalen van coloncarcinoma en levermetastasen. De opvallende uitbreiding van angiogene genexpressie tijdens tumorprogressie in stalen van onbehandelde patiënten kan een verklaring bieden voor de uiteenlopende resultaten van therapie gericht tegen een enkele VEGF alsook voor de zwakke tot matige efficiëntie van anti-angiogenese therapie.

Met het oog op de mogelijke sturende rol van eicosanoïden in de expressiesignaturen van de VEGFs, werd een gecorreleerde analyse uitgevoerd van de mRNA-expressie van *COX2*, *5-LOX* en *VEGFs*. Deze onthulde het complexe en verweven karakter van de inflammatoire en angiogene genexpressie reeds vanaf het adenoma stadium en tijdens verdere progressie naar het carcinoma stadium. Daarbij werden opvallende expressieprofielen van *5-LOX* en *COX2* gevonden in respectievelijk colonadenoma en -carcinoma en de aanwezigheid van deze enzymen was duidelijk geassocieerd met de cumulatieve expressie van VEGF familieleden. Tenslotte lijkt de dubbele expressie van *5-LOX* en *COX2* een verhoogd risico in te houden voor maligne transformatie van colonadenoma naar -carcinoma. Gezien de geobserveerde associatie tussen eicosanoïden en co-expressie van de VEGF familieleden, zouden colonkanker patiënten mogelijk voordeel halen uit een combinatietherapie die zowel de angiogene VEGF-signalisatie onderdrukt als de eicosanoïden om aldus de co-expressie van verschillende VEGFs te onderdrukken.

# Part I

# Introduction

# Chapter 1

# Colorectal cancer

Colorectal cancer is often referred to as a single disease although it is a collective term for cancer of the colon (colon/colonic cancer) and of the rectum (rectal cancer). Tumors located within 15 cm of the anal margin are classified as rectal while those more proximal are colonic [1]. Of those patients with colorectal cancer, approximately 35% are diagnosed with rectal and 65% with colon cancer [1, 2]. Although colon cancer is sometimes used as a synonym for colorectal cancer, throughout the thesis the following terms will be used when referring to these specific diseases: colorectal cancer for cancer of rectum and colon, colon cancer for cancer of the colon and rectal cancer for cancer of the rectum.

## 1.1 Facts and figures

Cancer is the third leading cause of death globally; preceded only by cardiovascular and infectious and parasitic diseases, and accounts for about 13% of all deaths per annum [3]. According to the most recent global study, approximately 7.6 million people died of cancer worldwide and 12.7 million people were diagnosed with invasive cancer in 2008 [3]. Colorectal cancer is worldwide the third and second most common cancer in men (10% of all cases) and women (9.4% of all cases), respectively (Figure 1.1) [3, 4]. In addition, colorectal cancer is the fourth most lethal type of cancer, accounting for 8% of all cancer deaths and with an estimated 608,700 deaths worldwide in 2008 [3].

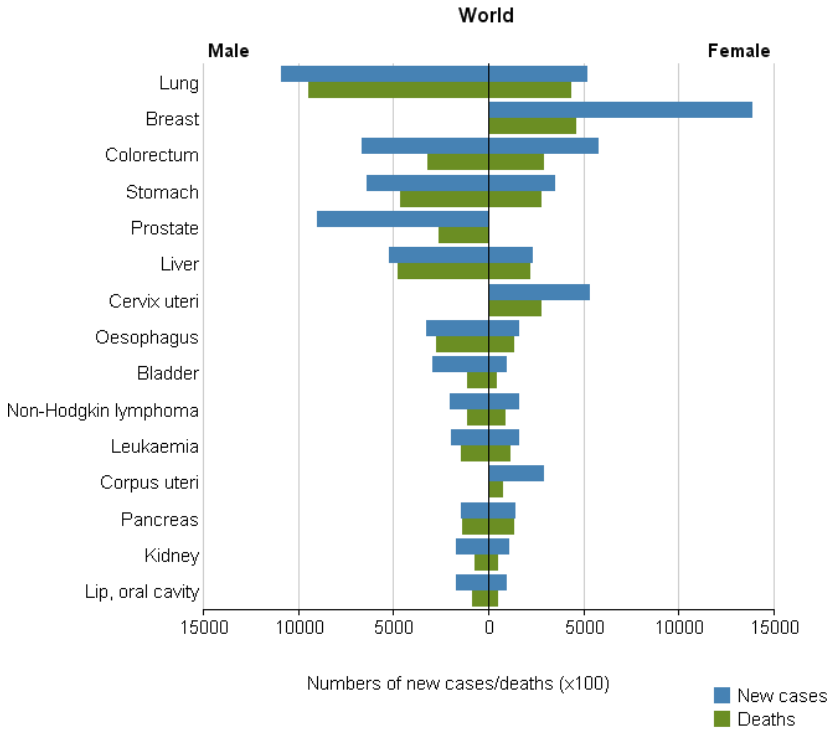



**Figure 1.1 - Estimated incidence and mortality rates of cancer worldwide.** Estimated age-standardized rates for the top 15 cancer sites. Source: Globocan 2008 [3].

Recent estimates for colorectal cancer predicted over 1.2 million new cases in 2008 [4]. Almost 60% of these cases occur in developed countries whereby the life-time risk for colorectal cancer is 5% for average-risk individuals in these industrialized regions [5]. The highest colorectal cancer incidence rates are observed in Australia and New Zealand, Western Europe and North America whereas the lowest incidence rates are found in Africa and South-Central Asia (Figure 1.2). Over the past two decades, incidence rates of colorectal cancer have stabilized in the majority of the economically developed countries, and have even declined in the United States as a result of national screening programs. However, incidence rates are rapidly rising in several historically low-risk areas including Spain and certain eastern-European and -Asian countries. The increase in these economically transitioning countries is probably due to their westernization including changing dietary patterns, obesity, physical inactivity, aging populations and increased smoking [4, 6, 7].

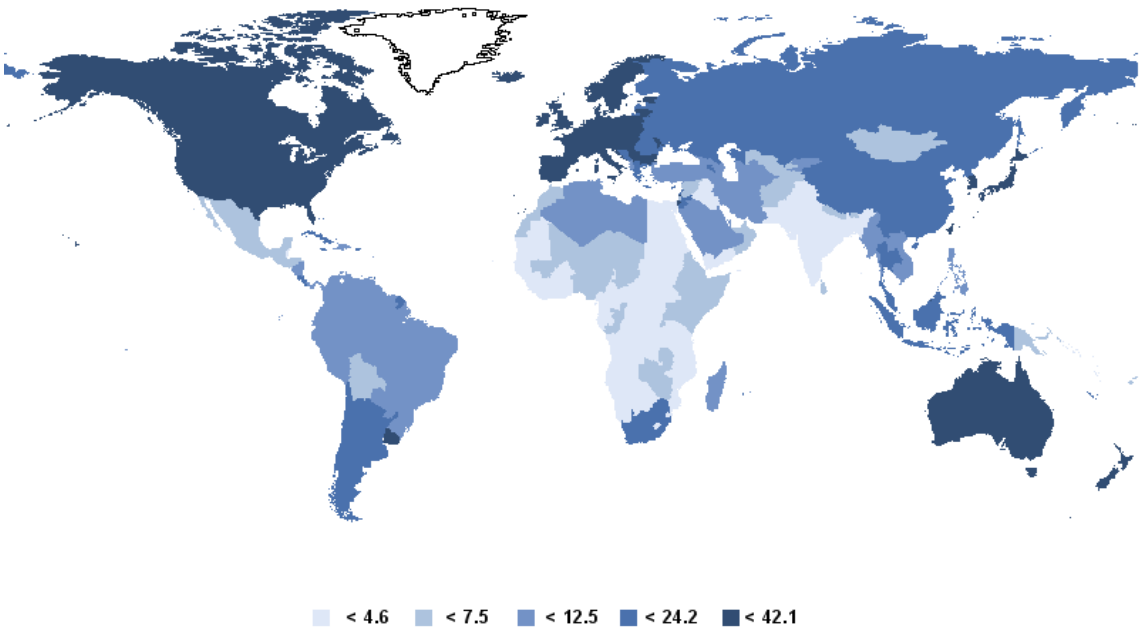



**Figure 1.2 - Estimated age-standardized incidence rate of colorectal cancer** per 100,000 inhabitants for male and female in 2008. Source: Globocan 2008 [3].

According to the latest data from the Belgian Cancer Registry, 8294 people were diagnosed with colorectal cancer in Belgium in 2010 [8]. The cumulative risk of developing colorectal cancer before the age of 75 years – in the absence of competing causes of death – was 5.1 and 3.2 % for Belgian men and woman, respectively [8]. In contrast to the United States, incidence rates of colorectal cancer have increased significantly for both sexes during the last decade, at least in the Flemish Region[1] (1.2% in males and 1.5% in females; Figure1.3). The mortality rates, however, have decreased significantly with 2.9% in males and 2.1% in females.

When comparing Belgium with countries exhibiting the highest incidence rates of colorectal cancer, Belgium ranks at eighth place in Europe, and twelfth place globally [3].

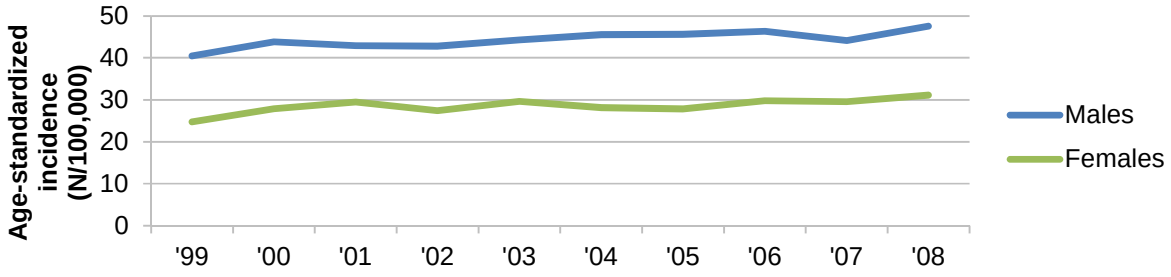



**Figure 1.3 – Age-standardized incidence of colorectal cancer in the Flemish Region, 2000-2010.** World standardized incidence rates per 100,000 persons for male and female from 2000 until 2010. Data were obtained from the Belgian Cancer Registry [8].

[1] 10 years registry data are not available for the Walloon Region.

## 1.2 Pathophysiology and progression

### 1.2.1 Precursor lesions

As demonstrated by epidemiologic, clinical, pathological, and molecular genetic findings, most cases of colorectal cancer develop from benign precursor lesions, i.e. adenomatous polyps or adenoma [5, 9, 10]. Premalignant adenoma evolves into malignant carcinoma through a series of genetic and epigenetic changes – the adenoma-carcinoma sequence – during a time interval of at least 10 years (Figure 1.4) [5, 10, 11]. Only ~5% of colon adenomas will eventually advance to the stage of carcinoma. This implies a significant biological difference between the formation of adenoma from healthy colon epithelium and the malignant transformation of colon adenomas into carcinomas [11]. The risk of transformation into colorectal cancer is believed to be affected by the number and size of polyps, the histological type and the presence of epithelial dysplasia [9, 10]. Premalignant colon adenomas arise from the lining of the intestine mostly by (epigenetic) silencing or DNA mutations in the APC tumor suppressor gene, disrupting the Wnt-signaling pathway [12].

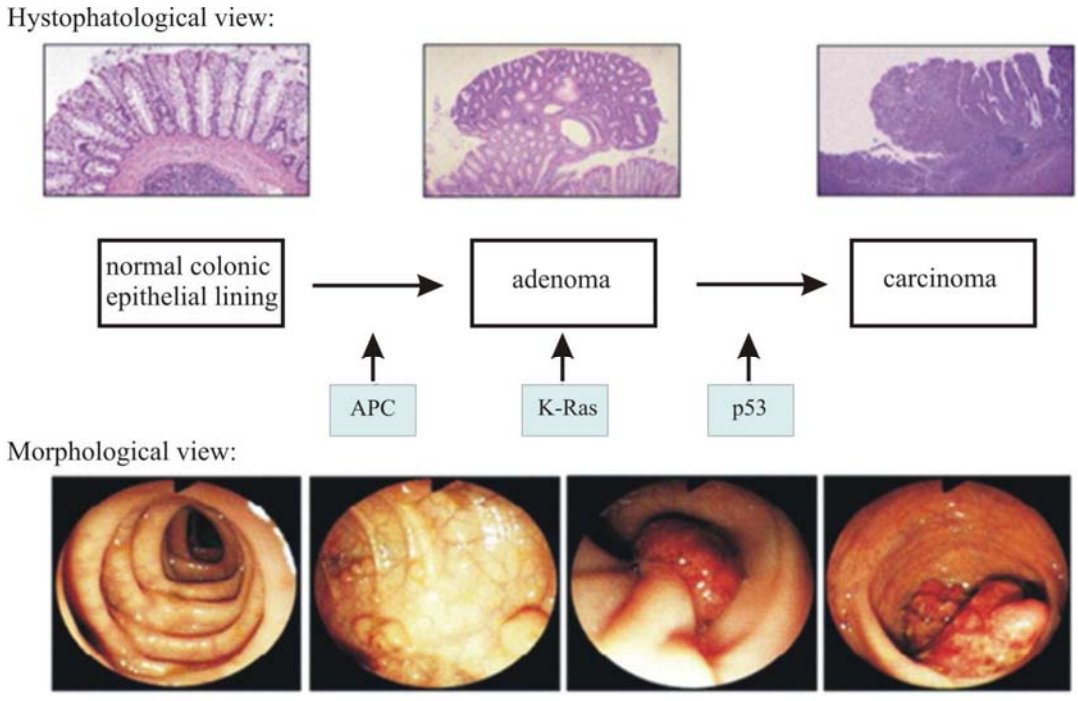



**Figure 1.4 -The adenoma-carcinoma sequence in colorectal cancer.** Histopathological and morphological (as viewed by colonoscopy) features of the initiation and progression of tumorigenesis from a normal colonic mucosa into an adenoma and a frank carcinoma with main associated molecular events. Adapted from Cardoso et al. [13]

Two widespread histologic types of polyps are discerned, namely serrated (including hyperplastic) and adenomatous polyps. 60 to 70% of all polyps removed at colonoscopy are adenomatous polyps or adenomas, the most common type [14]. They have typically hyperchromatically enlarged, cigar-shaped nuclei that are tightly packed in a palisade pattern and display varying levels of dysplasia [9]. Adenomas are classified as tubular, tubulovillous or villous. The most common adenomas are of the tubular type which histologically consists of tubular branches while the villous adenomas contain digitiform villi arranged in a cauliflower-like structure. In comparison, tubulovillous adenomas contain characteristic elements from both the tubular and villous types [9, 10]. High-risk or advanced adenomas are the most likely to become cancerous and either have a large size (i.e. >1cm), severe dysplasia or villous or tubulovillous characteristics [10, 15]. Adenomatous polyps give rise to 70 to 80% of all colorectal carcinomas.

The other 20 to 30% of colorectal carcinomas arise through the serrated pathways. Serrated polyps are characterized by their “saw-tooth” architectural features. Among the serrated lesions, hyperplastic polyps are the most common type but remain mostly small and localized to the distal part of the colon [16]. These polyps have serrations limited to the luminal outlines. An increased number of glandular cells with decreased cytoplasmic mucus but without nuclear hyperchromatism, stratification, or cytological atypia are histological references for hyperplastic polyps [15]. Hyperplastic polyps have generally little association with colorectal cancer [15, 17]. A link between the serrated pathway and colorectal cancer is presented by the sessile serrated lesions and traditional serrated adenomas, which generally arise within a hyperplastic polyp [9, 15]. Sessile serrated lesions are characterized by structural crypt abnormalities, like dilated crypt bases and lateral growth but the absence of cytological dysplasia. Traditional serrated adenomas on the other hand have the opposite characterizations, namely cytological dysplasia but a relative normal architecture [14, 15]. Filiform villi with stromal bulbous ends are typical for traditional serrated adenomas [15]. The characteristic alterations in the APC gene in conventional adenomas are absent in serrated polyps which often show BRAF (sessile serrated) and KRAS mutations (traditional serrated) and exhibit extensive DNA methylation, especially in DNA repair genes [9, 14]. Similar characteristics are observed in colorectal carcinoma with high microsatellite instability (MSI-H) arising from the serrated adenoma [9]. Table 1.1 summarizes the differences between sessile and traditional serrated adenomas, hyperplastic polyps, and adenomatous polyps.

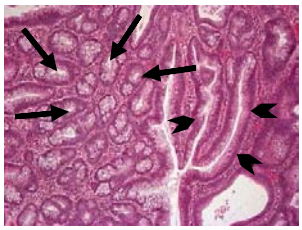
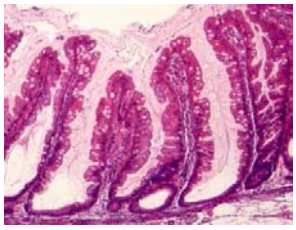
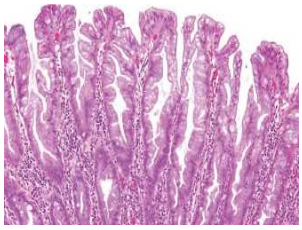
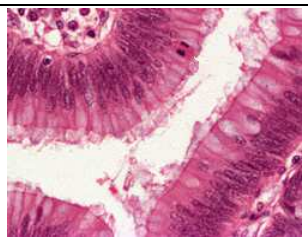
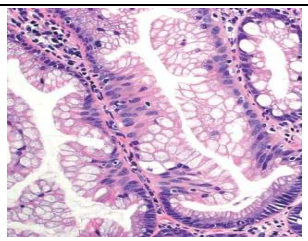
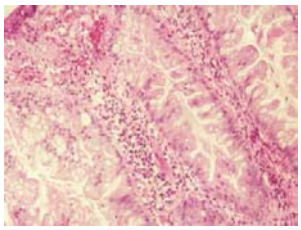
| | AP | SSA / TSA | HP |
|---|---|---|---|
| **Location** | Throughout, more right | Right colon / Left colon | Rectosigmoid |
| **Size** | Variable | Often ≥ 10 mm / < 10 mm | Small, often ≤5 mm |
| **Prevalence** | Extremely common | Common / Rare | Very Common |
| **Shape** | Pedunculated | Sessile, flat / Pedunculated | Sessile, flat |
| **Cytologic dysplasia** | Present | Minimal / Present | Absent |
| **Serration** | Absent | Present | Present |
| **Basal crypt dilation** | May be present | Present / Absent | Absent |
| **Horizontal crypts** | May be present | Present / Absent | Absent |
| **Branched crypts** | May be present | Present / Absent | Absent |
| **Ectopic crypts** | May be present | Absent / Present | Absent |
| **Basal serration** | Absent | Present | Absent |
| **Nuclear shape** | Tall columnar | Round to oval / Tall columnar | Flat or low columnar |
| **Precancerous** | Yest | Yes | No |
| **Low power microscopic view (~40x)** |  |  |  |
| **High power microscopic view (~200x)** |  |  |  |

**Table 1.1 - Comparison of colon polyp types.** The microscopic views of adenoma are derived from tubulovillous adenoma with tubular (arrows) as well as villous elements (arrowheads). The microscopic views of serrated adenoma are derived from sessile serrated lesions. AP: Adenomatous Polyp (tubular, tubulovillous and villous adenoma), SSA: Sessile Serrated Lesion, TSA: Traditional Serrated Adenoma HP: Hyperplastic Polyp. Pictures were obtained from Prof. Dr. K. Geboes [personal communication], Dr. M. Fleming [18] and Solunetti [19]. Table adapted from Freeman [16] and Makkar [14].

### 1.2.2 Hereditary colorectal cancer

Two genetic syndromes represent ~5% of colorectal cancer cases, namely familial adenomatous polyposis (FAP; ~1% of all colorectal cancers) and hereditary non-polyposis colorectal cancer (HNPCC or Lynch syndrome; 2-5% of all colorectal cancers). FAP is caused by a germline mutation in the APC gene and is characterized by hundreds of colon polyps [9, 10]. Unless the colon is removed, individuals with FAP will have developed cancer by the age of 40 [10]. HNPCC is caused by dominantly inherited germline mutations in DNA mismatch repair genes, mostly MLH1, MSH2 or MSH6. Individuals with HNPCC may develop colorectal cancer at an early age (average age of 45 years) and have an increased risk of malignancy at specific extracolonic locations including the endometrium, ovary, stomach and pancreas [10, 20]. The growth of multiple polyps is less often preceding the cancer but the developed adenomas contain more villous components and are more dysplastic. Furthermore, adenomas in HNPCC have an earlier onset and show an accelerated carcinogenesis (2-3 years *versus* $\geq$ 10 years) when compared to the general population [10, 20].

### 1.2.3 Sporadic colorectal cancer

Sporadic colorectal cancer is the result of a long-term (i.e. years to decades) multistep cascade of genetic and epigenetic mutations steering to disturbed DNA replication and accelerated mitosis in colonic cells. Eventually, progressive accumulation of genetic alterations leads to the adenoma-carcinoma sequence [9, 20]. Table 1.2 lists the most common molecular genetics of sporadic colorectal cancer.

### 1.2.4 Risk factors

Approximately 10-30% of colorectal carcinomas develop in persons with a clear family history of the disease but without defined disease-causing mutations [21]. Despite the lack of specific colorectal cancer-associated mutations in those families, these cancers are probably due to mutations in several genes in combination with environmental factors [10, 21]. In addition to genetic alterations and clear family history, environmental and demographic factors are also important elements in the etiology of colorectal cancer. The latter factors are presumed to modulate the risk of genetic mutations responsible for colon cancer although their specific mechanisms are currently unknown. The most prominent environmental factors are a sedentary

lifestyle concomitant with a high-fat diet, inadequate fiber intake and obesity, as well as alcoholism and smoking [9, 10, 20]. In addition, individuals with diabetes or inflammatory bowel disease, mainly ulcerative colitis and Crohn's disease, are at risk of developing colorectal cancer. For inflammatory bowel disease patients, the risk increases with the duration of illness (2% at 10 years; 18% by 30 years) and severity and extent of the inflammation [2, 10, 20].

| Gene | Chromosome location | Physiologic function of encoded protein | Clinical manifestations of mutation |
|---|---|---|---|
| **APC** | 5q | Regulates cell growth and apoptosis. | Mutated in 80-85% of sporadic colon cancer.<br>Homozygous somatic mutation associated with colon adenomas. |
| **K-ras family** | Various chromosomes | Encodes a small GTP-binding protein involved in transduction of mitogenic signals across cell membrane. | Mutated in ~50% of colon cancers.<br>May act in an intermediate stage of carcinogenesis.<br>Promotes colonocyte replication.<br>Mutation common in hyperplastic polyps. |
| **P53** | 17p | Regulates G1 cell cycle and apoptosis. | Mutated in ~75% of frank cancer.<br>Critical in transition from late adenoma to early cancer. |
| **DCC** | 18q | Encodes a neural cell adhesion molecule.<br>Facilitates apoptosis.<br>Tumor suppressor. | Believed to promote progression to frank carcinoma. |
| **Mismatch repair genes** | Various chromosomes | Recognize errors in nucleotide matching on complementary chromosome strand and initiate excision of erroneous strand. | Progressive accumulation of mutations throughout the genome in affected cells leading to hypermutability and genetic chaos. Mutations of oncogenes or tumor suppressor genes can lead to colon cancer. |

**Table 1.2 - Molecular genetics of sporadic colon cancer.** APC: adenomatous polyposis coli, DCC: deleted in colorectal cancer, GTP: Guanosine Triphosphate. Adapted from Cappell [9].

### 1.2.5 Metastasis

Colorectal cancer cells can spread to nearby lymph nodes causing local metastases or to remote organs forming distant metastases. Of all patients initially presented with colorectal cancer, approximately 20-30% is identified with synchronous metastases. An additional 20-30% of colorectal cancer patients will develop metachronous metastases over time [9, 10]. In colorectal cancer, the regional lymph nodes and the liver are the most common sites of metastases. Liver metastasis can occur early in colorectal cancer due to the venous drainage of the colon via the portal system straight to the liver. Approximately 75% of synchronous metastases in colorectal

cancer are liver metastases [22]. In the last few years, prognosis of liver metastases in colorectal cancer has improved substantially by the implementation of multidisciplinary management comprising chemotherapy and surgery [23]. Other sites of metastases become involved at a later stage and include the lungs, peritoneum, pelvis and adrenal glands [9, 10].

## 1.3 Diagnosis and treatment

### 1.3.1 Symptoms and presentation

The main symptoms of colorectal cancer are tiredness caused by anemia, weight loss, nausea, changes in bowel habits, melena or hematochezia and abdominal pain. However, these symptoms are also common with other pathological conditions [2, 24]. The cancer location and size and the presence of metastases determine the presented symptoms. Distal cancers may clearly produce rectal bleeding but this is not the case for proximal cancers. Because the blood is mixed with feces and chemically degrades during the passage through the colon, bleeding from proximal cancers is less obvious [9]. These patients then present anemia caused by iron deficiency instead of rectal bleeding. Symptoms associated with this type of anemia are weakness, fatigue, dyspnea or palpitations. Cancer cachexia can be present during the advanced stage, particularly when metastatic. The cachexia is characterized by poor health encompassing involuntary weight loss, anorexia and muscle weakness [9]. Occasionally, patients with colorectal cancer present as surgical emergencies with acute abdominal symptoms due to complications of the cancer [2, 25, 26]. Several complications can occur in colon cancer patients including bowel obstruction (8%-29% of cases), bowel perforation (2.5-10% of cases), abscess formation (0.3-4% of cases), acute appendicitis (3-25% of acute appendicitis cases) and ischemic colitis (1-11% of obstructive colon cases) [26].

### 1.3.2 Diagnosis, staging and prognosis

Diagnosis of colorectal cancer is based on the results of colonoscopy and pathologic examination of biopsies or removed polyps. The presence of metastases is generally imaged by CT of the chest, abdomen and pelvis. In addition, ultrasound and MRI determine the extent of the metastatic disease [9, 20].

After diagnosis, the colorectal cancer is staged according to either of two methods. The modified Dukes' staging system is based on postoperative findings while the tumor-node-metastases

(TNM) classification identifies the depth of tumor invasion (T), lymph node involvement (N) and presence of distant metastases (M). Invasive cancer in the TNM staging is classified from stage I to IV [9, 10, 20]. Table 1.3 summarizes the different classification systems in view of the stage dependent prognosis. If colorectal cancer is diagnosed at an early stage, the five-year survival exceeds 90%. But, in case of lymph node involvement, the five-year survival decreases to ~60% and below 10% if distal metastases are present [5]. However, early detection is challenging since clinical symptoms develop late in the progression of the disease [9].

| **TNM status** | | | **Stage** | **Modified Dukes' stage** | **5-years overall survival** |
|---|---|---|---|---|---|
| T *in situ* | N0 | M0 | 0 | - | likely to be normal |
| T1 | N0 | M0 | I | A | >90% |
| T2 | N0 | M0 | | B1 | 85% |
| T3 | N0 | M0 | IIa | B2 | 70-80% |
| T4 | N0 | M0 | IIb | B3 | |
| T1-2 | N1-2 | M0 | III | C1 | 25-60% |
| T3 | N1-2 | M0 | | C2 | |
| T4 | N1 | M0 | | C3 | |
| Any T | Any N | M1 | IV | D | 5-30% |

**Table 1.3 - Five-year survival rates by stage at diagnosis.** Adapted from Cooper et al. [10].

### 1.3.3 Screening

The asymptomatic nature of early colorectal cancer and its precursor lesions renders them hard to detect by clinical presentation. Therefore, screening of the population at risk (i.e. ≥ 50 years) is needed for early detection. Early diagnosis is crucial for increasing the chance at survival of colorectal cancer patients. In addition, timely removal of adenomas may decrease colorectal cancer incidence and mortality rate [27]. Colorectal cancer is an excellent candidate for screening because the disease has a high prevalence with recognized precursors and a clear benefit from early treatment [5]. However, screening for colorectal cancer is hindered by a lack of patient-friendly and affordable detection methods. Existing serum markers are currently restricted from use since they lack sufficient specificity and sensitivity. The most common serum marker, carcinoembryonic antigen (CEA), has mostly been used for postoperative surveillance [28]. Fecal

occult blood testing, notwithstanding it being a widely used screening modality, exhibits disadvantages like low sensitivity and specificity for carcinomas, and particularly for adenomas [27, 28]. Other stool-based tests which detect fecal DNA markers (e.g. K-ras, APC, p53) perform better, but are relatively expensive and far more laborious [28]. Tissue-based markers have been widely studied but rather for their potential prognostic and predictive value than for screening purposes. Currently, the gold standard for detection of colon carcinoma and adenoma remains endoscopic examination by colonoscopy. The main advantage of colonoscopy is that adenomas and early carcinomas can be removed immediately whereas blood and fecal based tests require colonoscopy afterwards for confirmation [27]. However, colonoscopy is an invasive procedure that requires hospitalization and anesthesia and carries the risk of complications [5]. This strongly impedes the broad application of colonoscopy as a screening tool because it renders the method particularly unpopular and unfit for large-scale screening.

### 1.3.4 Treatment

Colorectal cancer treatment is either curative or palliative. This is largely dependent on the location of the carcinoma, the extent of invasion into the bowel, and the infiltration into other organs. The treatment strategy therefore differs significantly for colon as compared to rectal cancer.

The current curative therapy for **rectal cancer**, both non-metastatic and metastatic, is based on combinations of chemotherapy, radiation therapy and total mesorectal excision (TME)-surgery. The preferred strategy comprises surgery preceded by neoadjuvant chemoradiation to reduce the size of the carcinoma and to facilitate effective surgery [20]. Radiation, combined with either 5-fluorouracil (5-FU) or capecitabine (Xeloda), is most commonly used as a preoperative therapy [29]. In case of metastatic rectal cancer, the metastatic lesion(s) may be resected simultaneously with the primary tumor or post-surgery [30]. The recommended adjuvant therapy is FOLFOX (infused 5-FU, leucovorin i.e. folinic acid and oxaliplatin) although the use of adjuvant chemotherapy following preoperative chemoradiation and surgery has not yet been optimally defined [29].

In contrast to rectal cancer patients, **non-metastatic colon cancer** patients receive no neoadjuvant therapy and their primary treatment is based on direct surgery followed by adjuvant

chemotherapy [30, 31]. Generally, early colon cancer, i.e. Tis/T1 N0 M0, is removed by polypectomy and does not require adjuvant therapy. For colon cancer stage T2 N0 M0 and higher, wide surgical resection with anastomosis is compulsory. Adequate distal margins of 5 cm or more are recommended and lymphadenectomy of at least 12 lymph nodes is needed for proper node staging [20, 30]. Due to small gains in survival, adjuvant therapy is controversial in stage II colon cancer patients and is considered on an individual basis. However, high-risk stage II patients[2] and especially stage III patients benefit from adjuvant therapy. Also here, infused FOLFOX is the standard adjuvant treatment [20, 29, 30].

The treatment of **advanced colon cancer** with synchronous metastases is mostly based on achieving R0 resection (i.e. microscopically cancer-free at the resection margins) of the primary tumor and metastases either before or after preoperative conversion treatment (Figure 1.5) [23, 30]. The basic strategy for treatment of colon cancer with synchronous metastases is summarized in figure 1.5. The treatment of advanced colon cancer comprises various active drugs, either as single agents or in combination. Basic cytotoxic chemotherapeutic agents which inhibit DNA replication are 5-FU/leucovorin, capecitabine, irinotecan and oxaliplatin [20, 23, 30, 31]. Recent progress in molecular biology has prompted the development of novel targeted therapies. Three of them are currently included in the first line treatment of metastatic colon cancer in combination with cytotoxic chemotherapy. Bevacizumab is a humanized monoclonal antibody against vascular endothelial growth factor A (VEGF-A), whereas the monoclonal antibodies cetuximab and panitumumab act by targeting the epidermal growth factor receptor (EGFR) [20, 23, 30, 31]. These targeted therapies are discussed in chapter 3 (3.4 Anti-angiogenesis therapy).

[2] High-risk stage II patients have T4 tumors, vascular/lymphatic/perineural invasion, colonic obstruction or perforation, fewer than 12 harvested lymph nodes or indeterminate/positive resection margins.

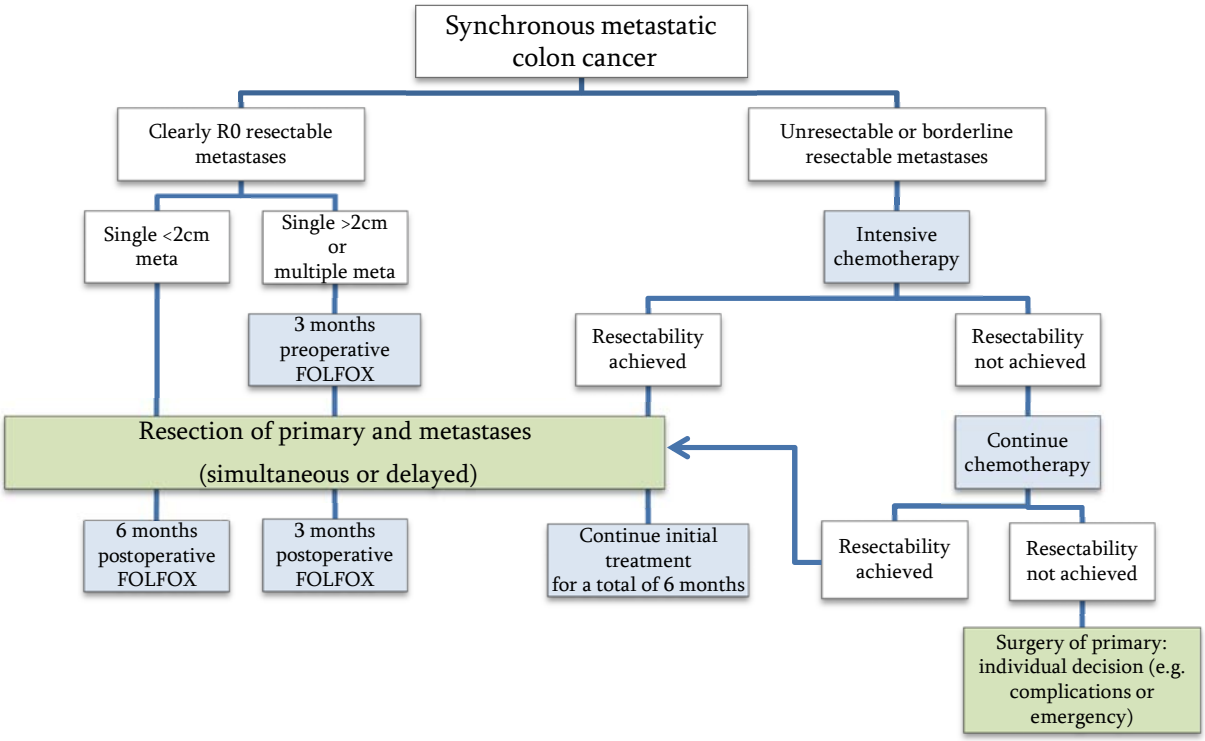



**Figure 1.5 - Treatment algorithm for synchronous metastatic colon cancer.** Adapted from Shmoll et al. [30]

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# Chapter 2

# Inflammation and cancer: encounters at the eicosanoid level

## 2.1 Introduction

The connection between inflammation and cancer has already been described some 5000 years ago in Ayurveda[1]. The characteristics and consequences of inflammation were firstly described as "redness (rubor), swelling (tumor), heat (calor) and pain (dolor)" in the first century by Aulus Cornelius Celsus, who was a Roman medical writer. In the 19$^{th}$ century, Rodolf Virchow postulated that inflammation caused by irritation leads to the development of most chronic diseases, including cancer. Yet only in recent years has this association been recognized as a generally accepted paradigm [1-3]. Colotta and colleagues have even postulated that inflammation is the seventh hallmark of cancer (Figure 2.1) [2].

The link between inflammation and cancer is clearly illustrated by the increased risk of cancer development in patients suffering from chronic inflammatory conditions. For example, cervical infection by human papillomavirus may lead to cervical cancer; hepatitis B and C or alcoholic liver cirrhosis can cause the development of hepatic cancer; and inflammatory bowel disease increases the risk of colon cancer [3, 4]. Physical, chemical or infectious tissue damage provokes a coordinated inflammatory response to eliminate the responsible agent and to initiate tissue repair. Inflammation should hence abate after elimination of the pathogen and promotion of wound healing. However, failure in the meticulous control of the immune response may result in an unresolved inflammation that continuously stirs the microenvironment. This can lead to a persistent induction of cell proliferation and tissue repair by alterations in cancer-related genes and posttranslational modifications in signaling proteins involved in cell cycle control, DNA repair and apoptosis [3]. The development of neoplastic cells is further encouraged by an environment with abundant inflammatory cells, pro-inflammatory cytokines and chemokines, and growth and survival factors [5].

[1] Ayurveda means "the science of long live". It is an ancient healing system that originated in India.

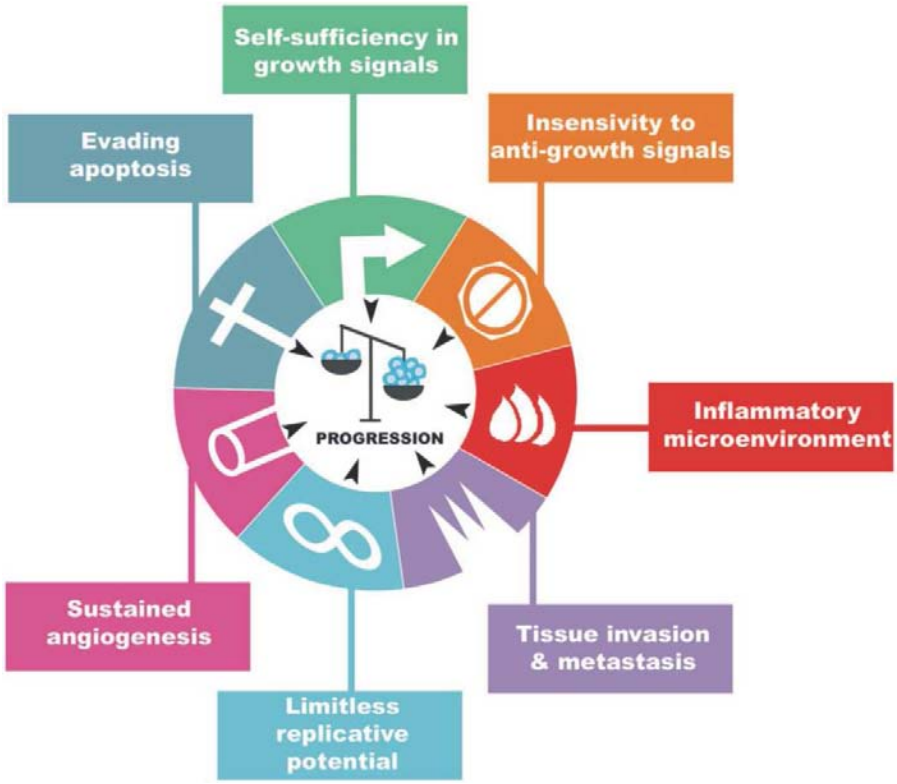


**Figure 2.1 - Inflammation as the seventh hallmark integrated in the six hallmarks of cancer** from Hanahan and Weinberg. Figure from Colotta et al. [2].

Endogenous promoters involved in cancer related inflammation are inflammatory transcription factors such as nuclear factor-kappa B (NF-κB) and signal transducer activator of transcription 3 (STAT3), as well as primary inflammatory cytokines such as interleukin (IL)-1α/β, IL-6 and tumor necrosis factor (TNF) [1-3]. NF-κB plays a central role in inflammation and carcinogenesis as evidenced by its constitutive activation in many cancers [1, 2]. Figure 2.2 summarizes NF-κB related inflammatory networking in cancer. In tumor, epithelial and immune cells, NF-κB activates the expression of genes encoding inflammatory cytokines, adhesion molecules, enzymes from the eicosanoid synthesis pathway, angiogenic factors and inducible nitric oxide synthase (iNOS). These NF-κB-activated factors may contribute to various processes of cancer development and maintenance like survival, transformation, angiogenesis, proliferation, invasion and metastasis. Inflammation-induced factors cyclooxygenase 2 (COX2) and 5-lipoxygenase (5-LOX), involved in eicosanoid synthesis pathways, and vascular endothelial growth factor (VEGF), contributing to (neo)-angiogenesis, will be discussed in detail in Chapters 2 and 3 respectively.

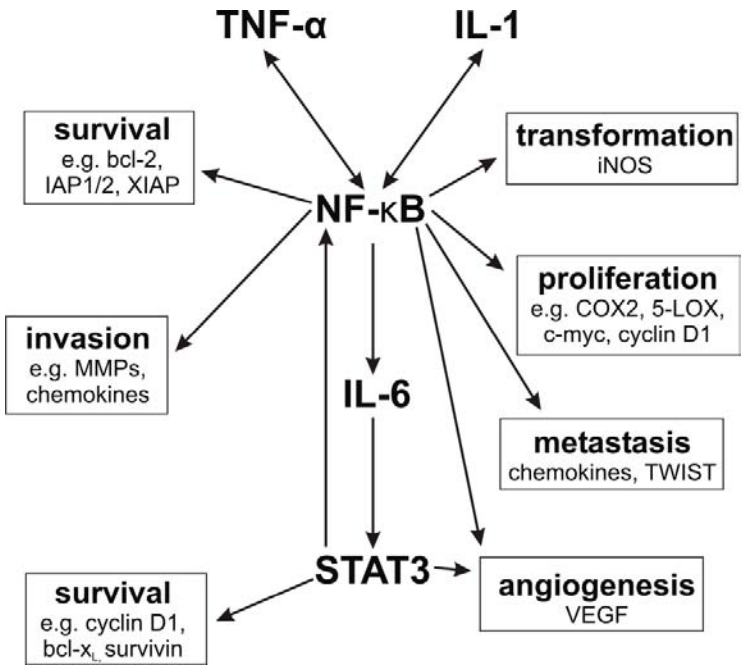


**Figure 2.2 - Inflammatory interactions in cancer orchestrated by NF-κB.** 5-LOX: 5-lipoxygenase, bcl-2/$X_L$: B-cell lymphoma 2/extra-large, COX2: cyclooxygenase 2, IAP1/2: inhibitor of apoptosis protein, iNOS: inducible nitric oxide synthase, MMPs: matrix metalloproteinase, NF-κB: nuclear factor-kappa B, TWIST: twist basic helix-loop-helix transcription factor, VEGF: vascular endothelial growth factor, XIAP: X-linked inhibitor of apoptosis protein. Adapted from Aggarwal et al. [1, 6]

## 2.2 Eicosanoid mediators in cancer

### 2.2.1 Eicosanoid synthesis pathways

Eicosanoids is a generic term for the biologically active lipids derived from arachidonic acid. Arachidonic acid is a polyunsaturated fatty acid present in the phospholipids of the cell membranes of mammalian cells. A major source of arachidonic acid is dietary animal fats. Eicosanoids, including prostanoids, leukotrienes, hydroxyeicosatetraenoic acids (HETEs), epoxyeicosatrienoic acids (EETs) and hydroperoxyeicosatetraenoic acids (HPETEs), are generated through the metabolism of arachidonic acid by cyclooxygenases (COX), lipoxygenases (LOX) and cytochrome P450 mono-oxygenases (Figure 2.3) [7]. In this chapter we will focus on the roles of cyclooxygenase, lipoxygenase and their main derivatives (i.e. prostanoids and leukotrienes) in sustaining established cancers.

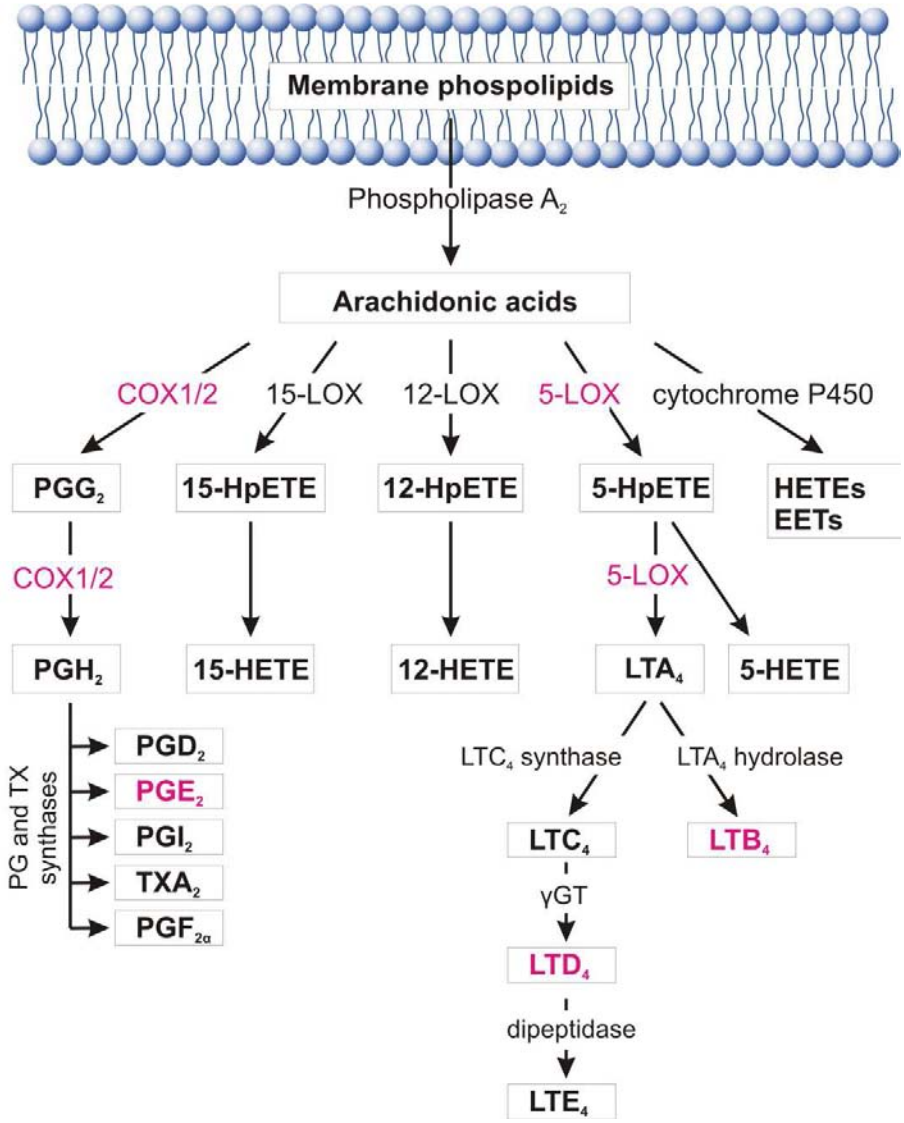


**Figure 2.3 - Overview of eicosanoid biosynthesis pathways**. Primarily the factors marked in red are discussed throughout the thesis. Adapted from Calder [8].

### 2.2.2 Cyclooxygenases in cancer

Cyclooxygenases catalyze the rate-limiting step of prostanoid synthesis by converting arachidonic acid into $PGH_2$, an intermediary metabolite used by specific prostaglandin and thromboxane synthases as substrate for prostaglandins (PG) and thromboxane $A_2$ ($TXA_2$) synthesis (Figure 2.3). The COX enzyme possesses two active sites, a cyclooxygenase site where arachidonic acid is converted into $PGG_2$, and a haem site with peroxidase activity that reduces $PGG_2$ to $PGH_2$ [9].

The COX enzyme family consists of three isoforms, namely COX1, COX2 and COX3. The most recently discovered **COX3** is a splice variant of COX1 in which intron 1 is retained [10]. COX3 has a low potency to generate prostanoids and therefore COX3 will not be further discussed. In general, **COX1** is constitutively present in most human cells and tissues and performs multiple housekeeping functions, including promoting platelet aggregation, vasoconstriction and protection of the inner stomach mucosae [9, 11]. Increased COX1 expression has been observed in various human cancers, including cervical [12], ovarian [13] and prostate cancer [14] and cancer of the head and neck [15]. **COX2** differs structurally from COX1 at a number of amino acids in the hydrophobic channel. This creates an extra side pocket whereupon the selectivity of COX2-inhibitors is based [16, 17]. In contrast to COX1, COX2 is an immediate-early response gene that is absent in most normal tissues except the kidney, colon and brain. COX2 is however induced strongly and rapidly in response to inflammatory cytokines, Toll-like receptors (TLR), hypoxia and tissue damage [11, 16, 18-20]. This rapid induction of COX2 generally subsides after 24 to 48 hours [21]. Constitutive overexpression of COX2 has been documented in various cancers, including gastrointestinal [22-27], genitourinary [28, 29], breast [30-32] and gynecologic cancers [33-35] as well as leukemia [36, 37] and head and neck cancers [38-40]. Besides in neoplastic cells in these tumors, COX2 is also expressed in stromal fibroblasts, infiltrating immune cells and endothelial cells of the tumor vasculature [19]. A key role of inducible COX2 in cancer has been demonstrated by numerous studies reflecting the pro-tumoral functions of the COX2-derived prostanoids (see 2.2.4. The contribution of eicosanoid mediators to colorectal cancer). The contributions of COX2 overexpression to the progression of colorectal cancer are summarized in figure 2.4. Overall, overexpression of COX2 in colorectal cancer is associated with lymph node and liver metastasis [41, 42], poor survival and prognosis [25, 43, 44] and tumor vascularization [41].

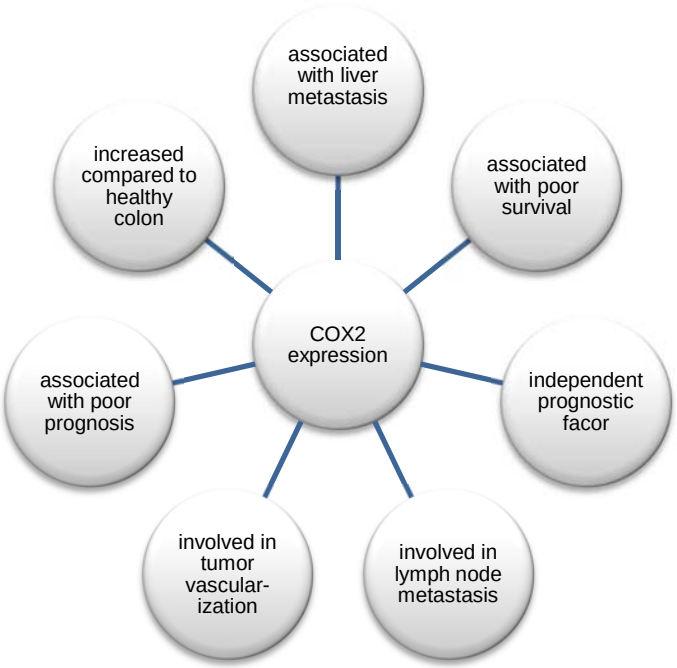


**Figure 2.4 - The effects of overexpression of COX2 in colorectal cancer patients.**

### 2.2.3 Lipoxygenases in cancer

Lipoxygenases catalyze the dioxygenation of arachidonic acid to a specific HPETE that is further metabolized to leukotrienes (LT) by 5-LOX, or reduced to the corresponding HETE (Figure 2.3) [45, 46]. The human LOX enzyme family consists of three groups classified according to their oxygenation position in arachidonic acid, namely 5-LOX, 12-LOX and 15-LOX. **5-LOX** converts arachidonic acid into 5-HPETE and metabolizes it further into $LTA_4$ [47]. Unique to 5-LOX is its dependence on $Ca^{2+}$ for translocation from the cytosol to the nuclear membrane, its linking to the transmembrane 5-LOX activating protein (FLAP) and its oxygenation and $LTA_4$ synthase activities [47, 48]. 5-LOX is generally found in cells of myeloid origin involved in inflammation and immune responses such as monocytes, macrophages, eosinophils, basophils, mast cells and B-lymphocytes [49]. However, increments of 5-LOX and its metabolites have been found in various types of cancers including prostate [50], bladder [51], breast [52], hepatocellular [53], colon [54], pancreatic [55], esophageal [56] and oral cancer [57]. This anomalous expression has been implicated in increased cell proliferation [58, 59], angiogenesis [60, 61] and resistance to apoptosis [56, 62]. In addition, 5-LOX overexpression has been observed in colon polyps [63, 64]. **12-LOX** has a distinct species-specific tissue distribution. In

humans, 12-LOX expression is found in platelets and the skin [65] but has also been detected in different cancers like prostate [66, 67], bladder [51], breast [30] and stomach cancer [68]. In addition, 12-LOX activity has been associated with tumor progression in prostate cancer [66]. The **15-LOX** enzyme is found in reticulocytes, eosinophils and the respiratory epithelium [46]. On this basis, the reticulocyte or leukocyte-type (i.e. 15-LOX-1) and the epidermis-type (i.e. 15-LOX-2) are discerned [69]. In addition to arachidonic acid-derived eicosanoids, 15-LOX-1 also produces 13-S-hydroxyoctadecadienoic acid (HODE) from linoleic acid [70]. Although the role of 15-LOX in cancer remains controversial, recent reports indicate the enzyme exerts anti-tumoral effects in line with its decreased expression in cancer. Thus, loss of 15-LOX-1 expression has been observed in major human cancers, including those of the colon [71], esophagus [72], breast [73], pancreas [74], bladder [75] and lungs [76]. Moreover, re-expression of 15-LOX-1 in colon and esophageal cancer has anti-tumoral effects [71, 72]. Similarly, 15-LOX-2 has been described as a functional tumor suppressor in prostate cancer [77], and to be decreased in esophageal [78] and breast cancer [73].

### 2.2.4 The contribution of eicosanoid mediators to colorectal cancer

The observed overexpression of COX1, COX2, 5-LOX and 12-LOX in various types of cancer results in an aberrant arachidonic acid metabolism and constitutively high levels of COX- and LOX-derived eicosanoids. These eicosanoids orchestrate complex interactions between tumor cells and the surrounding microenvironment through several mechanisms crucial for tumor survival, proliferation, progression, angiogenesis and metastasis. This section will focus on the effects of $PGE_2$ as predominant pro-tumoral prostanoid and of the leukotrienes $LTB_4$ and $LTD_4$, in sustaining colorectal cancer, summarized in figure 2.5.

#### *2.2.4.1 Tumor growth*

Deregulation of the balance between cell proliferation and cell death is the main hallmark of cancer. Enhanced cell divisions increase the number of cells while inhibition of apoptosis prolongs the survival of neoplastic cells and thus allows the further accumulation of genetic alterations. This leads to a process of natural selection that favors the multiplication and survival of genetically altered cells that facilitate neoplasia. Evidence from mouse models demonstrates that $PGE_2$ is a potent mediator of cell proliferation and survival in colorectal cancer. $PGE_2$ treatment in $Apc^{-/-}$ mice increases the adenoma burden, enhances epithelial cell proliferation, and

induces COX2 expression leading to a self-amplifying loop [79]. Also in azoxymethane-induced colon cancer, tumor incidence and multiplicity is enhanced by $PGE_2$ treatment [80]. On the other hand, deletion of prostaglandin E synthase (Ptges) and consequent inhibition of endogenous $PGE_2$ suppresses intestinal tumorigenesis in *Apc*$^{-/-}$ and azoxymethane models [81, 82]. $PGE_2$ induces proliferation by activation of the Ras-ERK pathway [79] and the β-catenin signaling pathway [83]. In addition to proliferation, $PGE_2$ promotes also survival of colon tumor cells in *Apc*$^{-/-}$ mice by activation of a PI3K-Akt-PPARδ cascade [84]. Moreover, an increased expression of the anti-apoptotic protein Bcl-2 has been associated with $PGE_2$ as well as $LTB_4$ and $LTD_4$ [85-87].

$LTD_4$ promotes survival of human intestinal epithelial cells by inducing the activation of the PKC-CREB and β-catenin signaling pathways as well as by preventing the activation of caspase 8 and the cleavage of BID [86, 88, 89]. LTB4, on the other hand, inhibits apoptosis of colon cancer cells via the activation of ERK and β-catenin signaling pathways [87, 90]. $LTB_4$ and $LTD_4$ are also involved in colorectal cancer cell proliferation as indicated by the suppression of proliferation in colon cancer cell lines by inhibition of either $LTB_4$ or $LTD_4$ [90-92]. Binding of $LTD_4$ to its $CysLT_1$ receptor regulates proliferation of intestinal epithelial cells by a PKC-mediated activation of the ERK1/2 pathway [88, 93] whereas $LTB_4$ induces cell proliferation in pancreatic cancer cells through MEK/ERK and PI-3 kinase/Akt pathways [94].

#### *2.2.4.2 Angiogenesis*

Tumor growth and metastasis is highly dependent on the tumor's ability to increase vascular supply for nutrient and oxygen delivery to growing tumor cells. Various cells in the tumor microenvironment, including tumor, stromal and immune cells, may contribute to tumor angiogenesis by the secretion of proangiogenic factors such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and platelet-derived growth factor (PDGF). These proangiogenic factors stimulate endothelial cell recruitment, proliferation, migration and tube formation. $PGE_2$ is an important factor in the induction of VEGF expression and synthesis. Binding of $PGE_2$ to the $EP_1$ receptor on colon cancer cells results in the activation of ERK. ERK activation subsequently leads to the translocation of hypoxia inducible factor-1 (HIF-1) to the nucleus and the induction of VEGF [95]. HIF-1, a major stimulus for angiogenesis, upregulates COX2 during hypoxia and hence increases $PGE_2$ levels. Elevated $PGE_2$ levels then further

enhance the expression of VEGF and transcriptional activity of HIF1 which in turn its contributes to COX2 expression. A positive feedback loop is therefore created which leads to COX2 and VEGF upregulation during hypoxia [20]. PGE2 also induces the expression of CXCL1 in colorectal cancer cells. CXCL1 is a pro-angiogenic chemokine that stimulates endothelial cell migration and tube formation [96]. The correlation between $PGE_2$ signaling and angiogenesis has also been described in stromal cells in mouse models of breast cancer and lung carcinoma [97, 98]. In endothelial cells, $PGE_2$ promotes αVβ3-integrin dependent migration and spreading, and induces VEGF and bFGF expression through stimulation of the ERK-JNK signaling pathway [99, 100]. In addition, $PGE_2$ also stimulates immune cells, such as mast cells to secrete VEGF and the pro-angiogenic monocyte chemoattractant protein-1 (MCP-1) chemokine [101, 102].

The contribution of leukotrienes to angiogenesis has been less well characterized. However, binding of $LTB_4$ to the BLT2 receptor on endothelial cells has been reported to stimulate VEGF-induced angiogenesis as well as endothelial cell migration and tube formation, thus indicating a role also for leukotrienes in neo-angiogenesis [103].

#### *2.2.4.3 Metastasis*

The progression of solid tumors relies on the ability of transformed cells to invade surrounding tissues (local metastasis) and to spread through blood or lymphatic vessels to distant tissues (distant metastasis). Metastasis is a multi-step process that displays a sensitive balance between host and tumor cell interactions. This process involves the invasion of tumor cells into the host stroma and blood vessels. Subsequently, tumor cells have to extravagate into the parenchyma of the new organ where they have to establish cell proliferation along with vascularization to form a metastatic focus [104]. Colorectal cancer cell migration and invasion is induced by $PGE_2$ through transactivation of epidermal growth factor receptor (EGFR)-PI3K-Akt signaling. EGFR transactivation is accomplished by the $PGE_2$-mediated induction of a β-arrestin1-Src kinase complex and stimulates colorectal cancer cell migration *in vitro* and metastatic spread to the liver *in vivo* [105]. $PGE_2$ treatment *in vitro*, up-regulates urokinase and matrix metalloproteinase (MMP)-9 expression via the JNK1/2 signaling pathway, thus causing degradation of the extracellular matrix and promoting cellular motility of human colon cancer cells [106].

Next to prostanoids, also leukotrienes may contribute to invasion and metastasis. As such, $LTD_4$ has been shown to trigger motility in non-transformed intestinal epithelial cells via a PI3K-Rac

signaling pathway [107]. In addition, the $LTB_4$ receptor-2 has been demonstrated to promote invasion and metastasis of ovarian cancer cells by a STAT3 upregulation of MMP-2 [108]. Although few in number, these studies indicate a role for leukotriene signaling in invasion and metastasis.

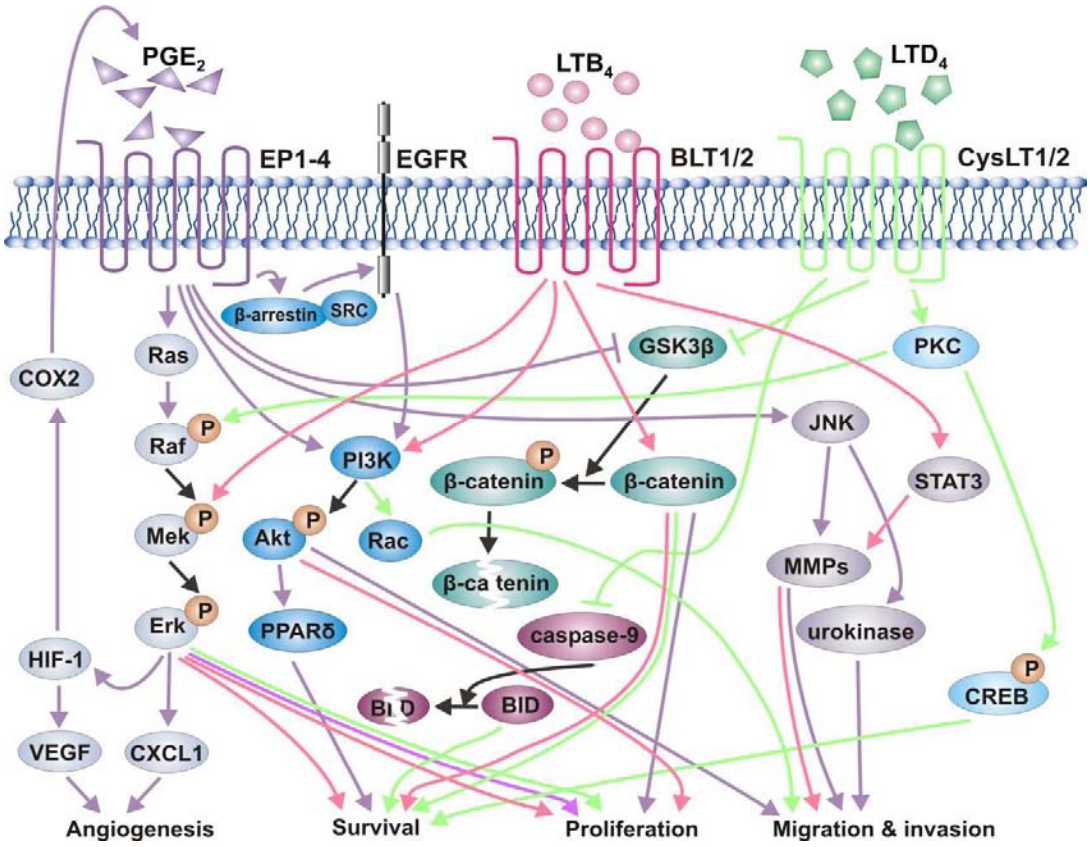


**Figure 2.5 – The promotion of cancer progression by $PGE_2$, $LTB_4$ and $LTD_4$.** Inspired by Wang [7].

## 2.2.5. Chemoprevention of colon cancer

Chemoprevention is the employment of pharmacological or nutritional agents to prevent, reverse or delay carcinogenesis before the development of invasive disease. Colorectal cancer is an excellent candidate for prevention since the progression to carcinoma after detection of an adenoma usually takes about a decade.

### *2.2.5.1 Nutritional agents*

Research into the advancement of chemopreventive agents is largely focused on natural agents targeting important carcinogenic pathways but without adverse side effects. Epidemiological,

experimental and clinical studies have investigated the potential anti-colorectal cancer activity of folic acid, calcium and micronutrient anti-oxidants like vitamin E and selenium [109]. In addition, nutrients like certain omega-3 polyunsaturated fatty acids (PUFAs), polyphenols and flavonoids exhibit inherent anti-inflammatory activity and have shown efficacy against colorectal carcinogenesis in rodent and *in vitro* models [109]. One such agent is eicosapentaenoic acid (EPA), a naturally occurring omega-3 PUFA with anti-inflammatory properties found in high quantity in oily fish. Dietary administration of EPA reduced colorectal tumor size and multiplicity in rodents [109] and showed reduced rectal polyp number and size in patients with FAP [110]. Furthermore the dietary administration of curcumin, a polyphenol derived from the spice tumeric, inhibited the development of azoxymethane -induced colonic premalignant lesions in an obesity-related colorectal carcinogenesis mouse model [111]. Inhibition of the production of $PGE_2$ and 5-HETE contributes to the anti-inflammatory and anti-carcinogenic properties of curcumin by direct inhibition of COX-1/2, Ptges, 5-LOX and the NFκB signaling pathway, [112, 113]. The Polyp Prevention Trial is a randomized dietary intervention trial examining the effectiveness of low-fat, high-fiber, high-fruit and high-vegetable diets on adenoma recurrence. Results from this trial associated a decreased risk of advanced adenoma recurrence with a high intake of flavonols, i.e. a class of flavonoids found in high concentration in beans, onions, apples and tea [114]. Again, inhibition of PGE2 by interference with COX2 may contribute to the anti-tumorigenic effects of flavonols [115]

#### *2.2.5.2 Pharmacological agents*

Aspirin and other non-steroidal anti-inflammatory drugs (NSAIDS), which are inhibitors of COX1 and/or COX2, are the most widely studied agents for chemoprevention of colorectal cancer. A decreased incidence of colorectal cancer for chronic aspirin users was observed for the first time in 1988 [116]. Since then , more than 100 randomized, double-blind, placebo-controlled animal studies have showed that NSAIDS consistently decreased the number of tumors per animal and animals with tumors [117]. In addition, multiple epidemiological studies (case-control and cohort) have reported a reduced colorectal cancer incidence and mortality, and adenoma development in men and woman treated with NSAIDS [117, 118]. The long-term use of traditional non-selective NSAIDs, however, is limited because it can cause serious gastrointestinal and renal side-effects. It has been shown that approximately 30% of daily

NSAIDs users develop gastrointestinal tract toxicity [119]. Traditional NSAIDs inhibit both the COX1 and COX2 isoforms and their toxicity is caused by the inhibition of COX1 housekeeping functions, namely the defense mechanisms of the gastrointestinal and renal mucosae by COX1-derived prostaglandins [117]. Therefore, specific COX2 inhibitors (COXIBs) were developed to obtain the beneficial effect of NSAIDs in the prevention of colorectal cancer but without their potential hazards. Several studies on colorectal cell lines treated with COXIBs, including meloxicam and celecoxib have shown the repression of neoplastic growth from the earliest stage of adenoma, i.e. the aberrant crypt foci [117, 120, 121]. These data were confirmed in animal models where COXIBs markedly reduced the number and size of azoxymethane -induced polyps and reduced the tumor burden up to 87% [117, 122-124]. The utility of COXIBs as chemopreventive agents was further demonstrated in about 40 observational studies in patients with FAP [125]. A 6 months placebo-controlled study of celecobix (Celebrex, Pfizer, New York) in FAP patients showed a 30% reduction in polyp burden [126]. Another study observed a 70%-100% reduction in the rate of polyp formation after treatment with rofecoxib (Vioxx, Merck, Whitehouse station, New Jersey) [127]. In 1999 and 2000, three randomized trials were launched in individuals with a recent history of adenomas to examine the role of different COXIBs for three years, namely APPROVe[2], APC[3] and PreSAP[4]. Despite the fact that all three clinical studies found a decreased incidence of adenomas, they were arrested early in 2004 because of cardiovascular toxicity [128-132]. A meta-analysis of the USPSTF[5] in 2007 confirmed the efficacy of COXIBs in the prevention of adenoma but did not recommend their use as a chemopreventive because of the cardiovascular risk, except for special groups at high risk of colorectal cancer [118]. For now, only celecoxib is approved by the regulatory agencies for chemoprevention in patients with FAP. For these patients, celecoxib is however merely used as a way to delay the time to surgery or as a secondary prevention therapy after prophylactic surgery. Thus, despite these promising experimental and clinical data, currently no chemopreventive strategy is available that replaces surgery and endoscopic surveillance.

[2] APPROVe: Adenomatous Polyp Prevention On Vioxx
[3] APC: Prevention of Sporadic Colorectal Adenomas With Celecoxib
[4] PreSAP: Prevention of Colorectal Sporadic Adenomatous Polyps
[5] USPSTF: U.S. Preventive Services Task Force

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# Chapter 3
# Wound healing and cancer: the angiogenic link

## 3.1 Wound healing

### 3.1.1 Introduction

Wound healing is a highly orchestrated process that is evolutionarily conserved and involves complex interactions of extracellular matrix (ECM) molecules, soluble mediators, various resident cells, and infiltrating immune cells. The healing process consists of three consecutive yet overlapping phases: inflammation with blood clotting, new tissue formation and tissue remodeling [1-4]. The onset of wound healing occurs immediately upon injury by the formation of a hemostatic blood clot with a platelet plug that seals the wound and initiates inflammation (Figure 3.1a). Inflammatory cells are attracted to the wound site by signaling of the activated platelets to prevent infection and remove debris. In addition, these inflammatory cells release growth factors, cytokines and proteinases, which initiate the phase of tissue formation [1-4]. During this phase, migrating fibroblasts proliferate and produce large amounts of ECM. This new tissue is called granulation tissue because of the granular appearance of the numerous capillaries that are sprouting at the wound edge as angiogenesis induces the development of new vasculature [3, 4]. In the final phase, granulation tissue is replaced by mature scar tissue through matrix remodeling. Eventually, the inflammatory response abates and the cellularity normalizes [3, 4].

Impaired wound healing represents a serious cause of morbidity and mortality, primarily affecting aged individuals (i.e. $\geq$ 65 years) and diabetic or immunosuppressed patients, as well as patients receiving chemo- or radiotherapy [3, 6]. Chronic wounds are, by definition, wounds that have failed to progress through the stepwise process of physiologic healing. Instead, they are trapped in a phase of pathologic inflammation that causes a delayed, incomplete and uncoordinated healing process and impairs the restoration of anatomic and functional integrity of the tissue [6, 7]. At the opposite end of the spectrum, excessive healing causes fibrosis with overabundant collagen deposition and reduced remodeling. This fibrosis results in loss of the anatomical structure of the tissue and thus compromises its function [8].

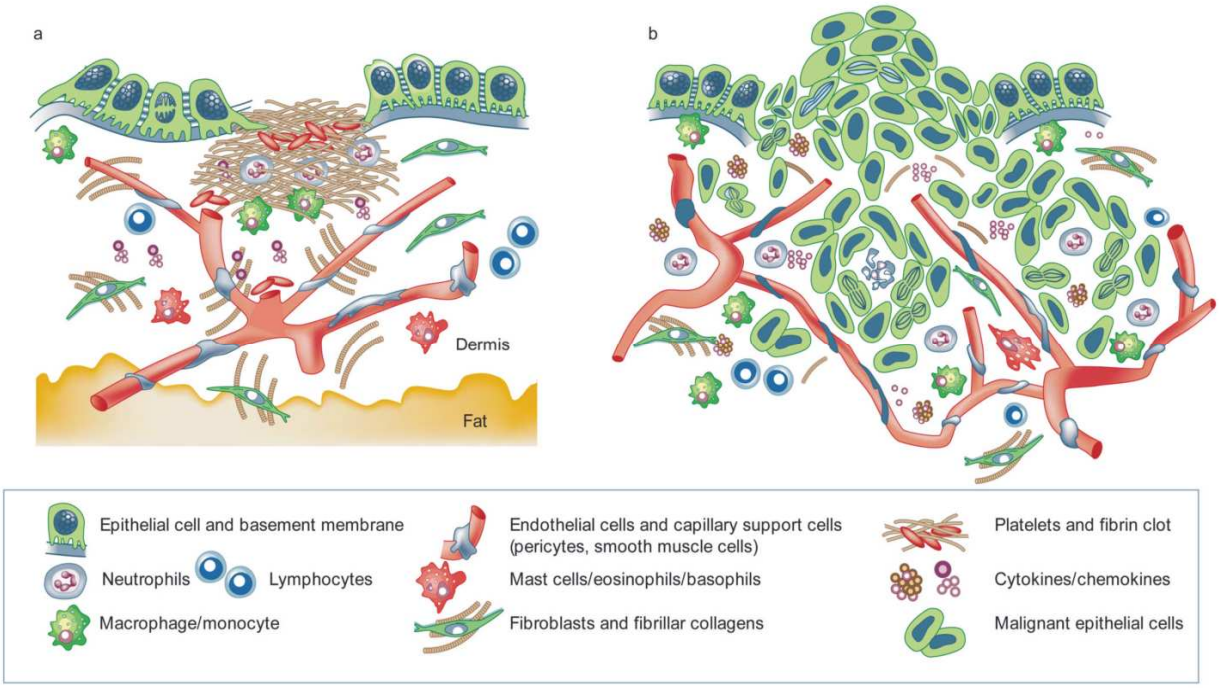


**Figure 3.1 - Wound healing versus invasive tumor growth.** ***a.*** Normal tissues have a highly organized structure with epithelial cells upon a basement membrane which separates them from the vascularized stromal compartment. Upon tissue damage, activated platelets form a hemostatic plug and release vasoactive factors that regulate vasoconstriction and formation of the fibrin clot. The activated platelets also release chemotactic factors like TGF-β and PDGF that initiate the formation of granulation tissue as well as the activation of fibroblasts and remodeling of the ECM. In addition, granulocytes and monocytes are recruited, and the venous network is restored. After re-epithelialization and healing of the wound, signaling subsides. ***b.*** Invasive carcinomas are less organized. The interaction of neoplastic cells with other cell types (i.e. mesenchymal, hematopoietic and lymphoid) causes the production of a chaotic vascular organization of blood and lymphatic vessels by angiogenesis an lymphangiogenesis. Neoplastic cells release cytokines and chemokines that are mitogenic and/or chemoattractants for granulocytes, mast cells, monocytes, macrophages, fibroblasts and endothelial cells. In return, these activated fibroblasts and inflammatory cells produce cytokines and chemokines that are mitogenic for neoplastic and endothelial cells.

### 3.1.2 “Cancers are wounds that do not heal”

Already in 1863 Rudolf Virchow proposed that malignant transformation represents the most severe complication of both impaired and excessive healing. He hypothesized that chronic irritation and previous injuries are a precondition for carcinogenesis [3]. The American pathologist Harald Dvorak then postulated in 1986 that “cancers are wounds that do not heal” [9]. He had recognized remarkable similarities between the granulation tissue of healing skin wounds and the composition of the stroma of malignant tumors. Therefore, he presumed that tumors activate the wound healing response of their host for the formation of the tumor stroma. Both postulations have been supported by numerous clinical observations and experimental studies [3].

| Hallmark | Cancer | Wound healing |
|---|---|---|
| **Proliferative signaling** | Sustained | Transient |
| **Evasion of growth suppression** | Sustained | Transient |
| **Cell migration** | With invasion and metastasis | Without invasion and metastasis |
| **Enabling replicative immortality** | Yes | No |
| **Angiogenesis** | Sustained | Transient |
| **Cell death** | Resisting | Transient increase |
| **Inflammation** | Sustained | Transient |

**Table 3.1 - Comparison between the hallmarks of cancer and wound healing.** Adapted from Arwert et al. [1].

Although many components of wound healing have been found in cancer, there are also important differences as illustrated in figure 3.1 and summarized in table 3.1. The most important difference is that wound healing is a self-limiting process whereas cancer is not [1]. This difference is caused by a dissimilar expression and activation of microenvironmental factors [1, 3]. Thus, the released growth factors, cytokines and chemokines show striking similarities between healing wounds and tumors [10]. However, these factors differ in the kinetics of their expression and become constitutively activated in solid tumors (Table 3.2) [1]. Furthermore, vessels in wounds and tumors are initially immature and leaky. This hyperpermeability allows the continuous release of plasma proteins and the deposition of a fibrin and fibronectin matrix, typical for most cancers and for healing wounds. This leakiness of the vessels with the deposition of the matrix is an acute and transient event in wound healing, whereas it is a persistent event in most cancers [3].

## 3.2 Angiogenesis and lymphangiogenesis as part of tumor vascularization

### 3.2.1 Introduction

The formation of new blood vessels is crucial for the supply of oxygen and nutrients to the healing tissue as well as to growing solid tumors. Neovascularization has an essential role in the growth of tumors beyond a diameter of 2mm and in metastasis [11]. Angiogenesis is the predominant approach of neovascularization in wound healing as well as in tumors [3]. Upon the onset of angiogenesis in healing wounds, also lymphangiogenesis is initiated in order to reconstruct the lymphatic vasculature. This process is particularly important for metastasis in tumorigenesis [12].

| | **Functions in wounds** | **Functions in cancer** |
|---|---|---|
| Growth Factors | | |
| EGF family | Epidermal and mesenchymal regeneration; accelerates wound healing | Cancer cell invasion, macrophage signaling and autocrine growth of tumor cells |
| FGF family | Early angiogenesis, fibroblast proliferation and re-epithelialization via keratinocyte migration | Angiogenesis and fibroblast proliferation |
| TGFβ family | Attracts neutrophils and macrophages, mediates ECM deposition, angiogenesis, epithelial cell migration and wound healing | Tumor development, tumor cell invasion and metastasis |
| PDGF | Attracts neutrophils and macrophages, and mediates ECM deposition and angiogenesis. | Recruits inflammatory cell infiltration and mediates angiogenesis and lymphangiogenesis |
| VEGF | Angiogenesis | Tumor cell invasion and angiogenesis |
| Cytokines and chemokines | | |
| IL-1α/β | Fibroblast and keratinocyte proliferation and neutrophil recruitment | Tumor cell proliferation, angiogenesis and inflammation |
| IL-6 | Fibroblast proliferation and neutrophil recruitment | Tumor development, tumor cell invasion and metastasis |
| TNF | Leukocyte infiltration | Tumor promotion or suppression |
| CSF1 | Recruitment of macrophages and re-epithelialization | Tumor cell invasion and migration |
| MCP1 | Macrophage recruitment, angiogenesis, re-epithelialization and ECM production | Monocyte recruitment, tumor cell invasion and metastasis |
| CXCL1 | Neutrophil infiltration, epithelial migration and neovascularization | Angiogenesis, invasion and migration |
| CXCL2 | Epithelial proliferation | Recruits inflammatory cell infiltration and migration |
| CXCL8 (also known as IL-8) | Inflammation, wound contraction and epithelial proliferation | Angiogenesis, migration and invasion |
| CXCL12 | Angiogenesis | Migration, invasion and angiogenesis |

**Table 3.2 - Cytokines, chemokines and growth factors that influence wound healing and tumor progression.** Cytokines, chemokines and growth factors are included on the basis that they have been shown to influence both wound healing and tumor invasion or progression in vivo. CSF: colony stimulating factor, CXCL: C-X-C motif ligand, EGF: epidermal growth factor, FGF: fibroblast growth factor, IL: interleukin, PDGF: platelet-derived growth factor, MCP1: monocyte chemoattractant protein 1, TGF: transforming growth factor, TNF: tumor necrosis factor, VEGF: vascular endothelial growth factor. Adapted from Arwert et al. [1].

### 3.2.2 Tumor vascularization

Autopsies of individuals who died from non-cancer causes has led to the discovery of dormant tumors. Initially, tumors grow avascular, until an equilibrium between proliferation and apoptosis is reached. Unless they progress to a vascularized growth, these tumors persist in a dormant encapsulated state [13]. Vascularized tumors are associated with a poor prognostic outcome because vascularization is correlated with aggressiveness and metastasis [14]. The growth of

tumor vessels is a hallmark of cancer since it is an essential adaptation of the microenvironment for the development of clinically relevant tumors [15]. The tumor associated vascularization exploits canonical mechanisms of physiological vessel growth, yet in a deregulated manner. However, tumors may also develop additional strategies to ensure their blood supply. As a result, tumor vascularization may be heterogeneous and muddled [13]. Angiogenesis is defined as the formation of new blood vessels from pre-existing vessels. This can be achieved by either sprouting angiogenesis or intussusceptive angiogenesis. Sprouting angiogenesis involves proliferation and migration of endothelial cells (Figure 3.2.A). It starts by degradation of the ECM and the basement membrane surrounding the endothelial cells by activated proteases. Hence, the endothelial cells are capable to invade the surrounding matrix, to proliferate and to migrate through the matrix [13, 16]. Intussusceptive angiogenesis is faster than sprouting angiogenesis since it does not primarily depend on the proliferation of endothelial cells [17]. Instead of endothelial cell proliferation, the capillary network increases its complexity and vascular surface through the insertion of multiple transcapillary pillars of connective tissue to partition the vessel lumen (Figure 3.2.C) [17]. Besides angiogenesis, the recruitment of bone marrow-derived cells (BMDCs) can also contribute to tumor vascularization (Figure 3.2.B). The majority of these BMDCs (e.g. neutrophils, leukocytes and tumor associated macrophages) are not physically incorporated in the vessel wall but gather in the tumor microenvironment to sustain angiogenesis [18]. Angioblasts (i.e. endothelial progenitor cells) on the other hand, can actually form blood vessels together with endothelial cells [16]. Vasculogenic mimicry is another way of vascularization that occurs mainly in aggressive tumors (Figure 3.2.D). It is a process of cell plasticity in which tumor cells dedifferentiate and gain the ability to form vessel-like structures [13, 16]. In addition, cooption can be used for vascularization by tumors in highly vascularized tissues, like the brain and the lungs (Figure 3.2.E). Hereby, tumor cells grow alongside pre-existing vessels at the early stage of tumor development. As the tumor progresses, apoptosis of the coopted endothelial cells is induced, provoking hypoxia and angiogenesis [13, 16].

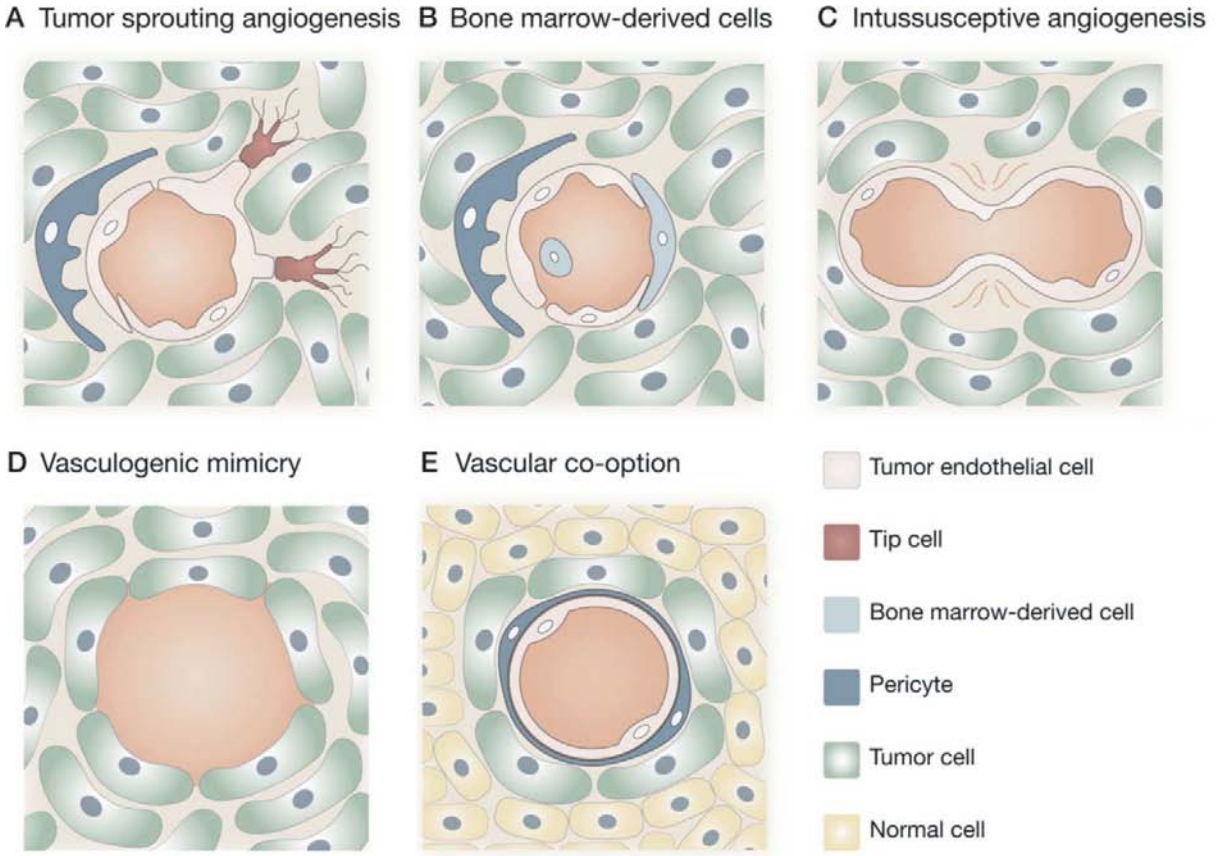


**Figure 3.2 - Representation of the different mechanisms involved in tumor vascularization.** Tumor vascularization may occur through five different mechanisms. These mechanisms are: ***(A)*** tumor sprouting angiogenesis, ***(B)*** bone marrow-derived cells, ***(C)*** intussusceptive angiogenesis, ***(D)*** vasculogenic mimicry and ***(E)*** vascular co-option. Figure from de Oliveira et al. [13].

The tumor vasculature is characterized by its atypical morphology with irregularly shaped and tortuous vessels that lack the normal hierarchical arrangement of arterioles, capillaries and venules (Figure 3.3). The endothelial cells in tumor vessels show an anomalous gene expression and require growth factors for their survival. Furthermore, the cells don't form tight monolayers like in normal blood vessels, resulting in a leaky vasculature [19, 20]. Together with pericytes, these aberrant endothelial cells generate a defective basement membrane, which contributes to tumor cell invasion. The basement membrane of tumor blood vessels has an irregular thickness as well as focal holes and broad extensions into the tumor stroma [19, 20]. In addition, the basement membrane and the pericytes are only loosely attached to the endothelial cells, which may weaken the blood vessels and increase the risk of hemorrhage [19, 20].

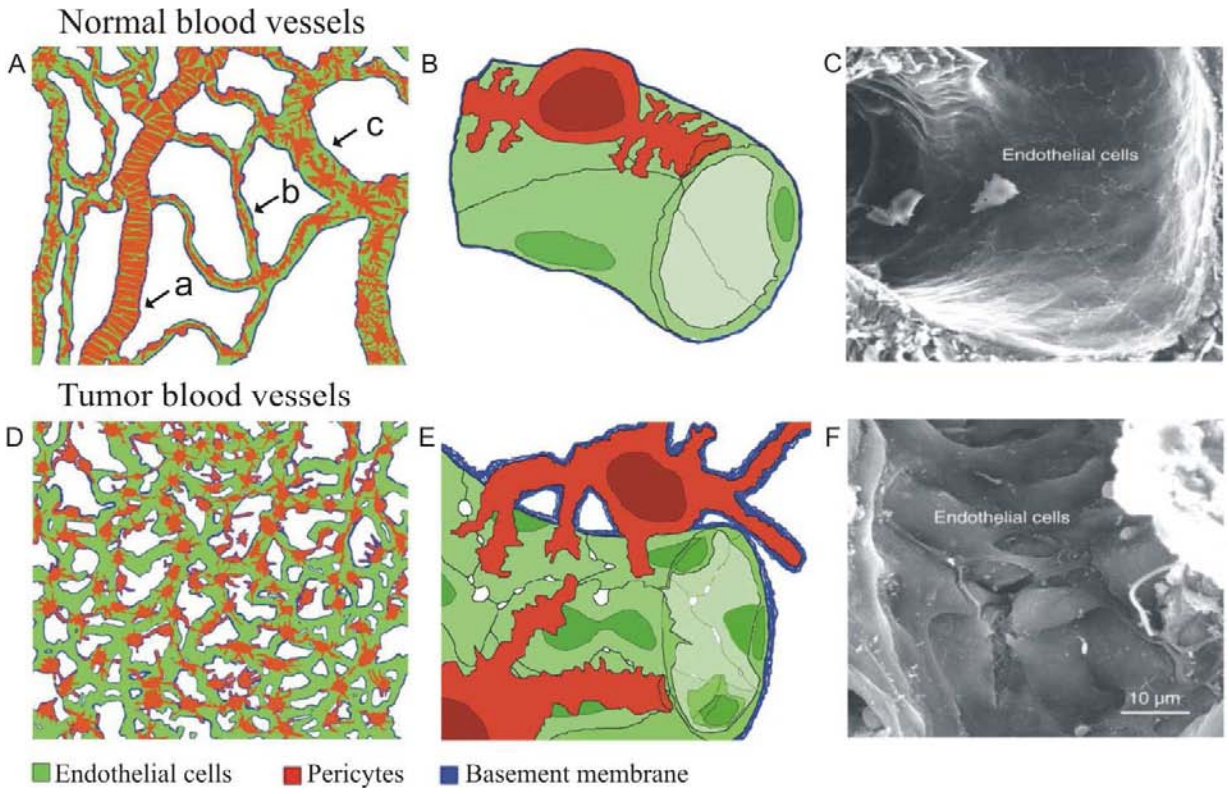


**Figure 3.3 - Comparison between normal and tumor blood vessels.** A, B, D and E are schematic diagrams, at two magnifications, comparing the vasculature of a normal organ with that of a tumor. All blood vessels consists of endothelial cells (green), pericytes (red) and vascular basement membrane (blue). ***(A-B)*** The normal vasculature shows a hierarchy of arterioles (a), capillaries (b) and venules (c). Pericytes are wrapped around the arterioles and accompany the capillaries and venules. The thin basement membrane surrounds all mural and endothelial cells. ***(D-E)*** Tumor blood vessels show a disorganized, anastomotic network of vessels, lacking the normal hierarchy. Gaps are present between the endothelial cells. Pericytes are loosely associated with endothelial cells and have irregular shapes. The basement membrane has multiple layers in some places. Scanning electron micrographs ***(C)*** of normal blood vessels where a smooth and tight endothelial cell monolayer covers the luminal surface of the vessel and ***(F)*** of the disorganized endothelium of a blood vessel in a RIP-Tag2 tumor. Adapted from Baluk et al. [19].

### 3.2.3 Lymphangiogenesis in cancer

Besides angiogenesis, also lymphangiogenesis occurs during inflammation, wound healing and carcinogenesis. The lymphatic network is an open ended, one way transport system, that drains extravasated fluid, collects lymphocytes and returns it to the blood circulation. There is accumulating evidence that the lymphatic systems plays a role in tumor progression. One of the early signs of malignant cancer spread is the metastasis to regional lymph nodes. This event represents the first step of tumor dissemination for many human cancers, including breast, colon and prostate cancer [21]. From the lymphatic system, cancer cells can disseminate to other tissues [16]. Tumor lymphangiogenesis has received less scientific attention than tumor angiogenesis

due to the lack of specific markers for lymphatic vessel endothelial cells (LVECs). Recent improvements of isolation techniques for LVECs has led to the discovery of LVEC markers like vascular endothelial growth factor receptor-3 (VEGFR-3), lymphatic vessel endothelial hyaluronan receptor -1 (LYVE-1), podoplanin and Prox1 [16, 22]. However, the mechanism of lymphangiogenesis has not yet been determined. Neo-lymphatic vessels in tumors may originate from bone marrow-derived endothelial cells in a similar way as new blood vessels [23]. Other studies claimed that new intratumoral lymphatic vessels are formed by "budding" of pre-existing lymphatic vessels and even by transformation of other cells into LVECs [24, 25]. The newly formed intratumoral lymphatic vessels have similar structures than new physiological vessels but the intratumoral vessels have thinner walls and lack tight junctions, facilitating tumor metastasis [26]. Intratumoral lymphatic vessels have been associated with lymph node metastasis as well as local recurrence and poor prognosis. Yet, peritumoral lymphatic vessels are believed to play a more important role in tumor metastasis because they provide more channels for lymphatic invasion and metastatic spread [27].

## 3.3 Angiogenic mediators in cancer

### 3.3.1 Introduction

Physiological angiogenesis is initiated by ischemic and hypoxic signaling and is tightly regulated by a balance between pro- and anti-angiogenic factors. A shift in this balance in favor of pro-angiogenic molecules causes an upregulated and continuous active angiogenic network during tumor angiogenesis [20]. Table 3.3 summarizes various mediators involved in tumor vascularization. Some factors contribute to several mechanisms, indicating the collaboration of these mechanisms to warrant tumor vascularization. From this overview the vascular endothelial growth factor (VEGF) family members emerge as crucial mediators as they are involved in every mechanism of tumor vascularization.

| | |
|---|---|
| Sprouting angiogenesis | |
| VEGF-A | Induction of eNOS; increase of vessel permeability; production of proteases; stimulation of endothelial cell proliferation, migration and survival; stimulation of tube formation |
| PDGF-B | Stimulation of mural cell proliferation and migration |
| Ang1 | Stimulation of endothelial cell survival and migration; induction of vessel stabilization |
| Ang2 | Pericyte/endothelial cell interaction loosening; stimulation of endothelial cell proliferation |
| TGF-β | Production of proteases and ECM; stimulation of endothelial cell proliferation and migration; modulation of junctional adhesion molecules |
| VEGF-B | Stimulation of endothelial and mural cell survival |
| bFGF | Production of proteases; stimulation of endothelial cell proliferation; stabilization of newly formed vessels |
| PlGF | Regulation of VEGF-A-mediated angiogenic switch |
| IL-8 | Stimulation of endothelial cell migration |
| Intussusceptive angiogenesis | |
| VEGF-A | Formation of larger vessels and small holes in the capillary plexus |
| TGF-β | Increasing intussusception |
| Ang1 | Formation of larger vessels and small holes in the capillary plexus |
| Lymphangiogenesis | |
| VEGF-C | Stimulation of proliferation, migration and survival of lymphatic endothelial cells |
| VEGF-D | Induces dilation of collecting lymphatic vessels |
| Angioblast recruitment | |
| VEGF-A | Induction of MMP-9; mobilization of endothelial precursor cells from the bone marrow; mobilization of circulating endothelial precursor cells in blood; differentiation of multipotent adult progenitor cells into endothelial cell. |
| Ang1 | Mobilization of endothelial precursor cells from the bone marrow |
| PlGF | Mobilization of endothelial precursor cells from the bone marrow |
| SDF-1 | Mobilization of endothelial precursor cells from the bone marrow; activation of MMP-9 |
| Selectins, integrins | Adhesion of endothelial precursor cells to the vessel wall |
| Cooption | |
| VEGF-A | Induction of sprouting angiogenesis during remodeling |
| Ang2 | Induction of vessel degradation in absence of VEGF-A; induction of sprouting angiogenesis during remodeling (with VEGF-A) |
| Vasculogenic mimicry | |
| PI3K | Activation of MMP-2 |
| TFPI-2 | Formation of vasculogenic network; activation of MMP-2 |
| VEGF-A | Stimulation of tumor cell plasticity; induction of MMPs |
| VE-cadherin | Stimulation of MMP-2 and -9 |

**Table 3.3 - Factors contributing to tumor vascularization.** Ang: angiopoietin, bFGF: basic fibroblast growth factor, IL: interleukin, PDGF: platelet-derived growth factor, PlGF: placental growth factor, SDF-1: stromal cell-derived factor-1, PI3K: phosphatidylinositide 3 kinase, TFPI-2: tissue factor pathway inhibitor-2, TGF: transforming growth factor, VEGF: vascular endothelial growth factor. Adapted from Auguste et al [28] and supplemented with [16, 29-31].

### 3.3.2 The VEGF family

The human VEGF family consists of five members, namely VEGF-A, VEGF-B, VEGF-C, VEGF-D and placental growth factor (PlGF). The VEGF family of ligands interacts with three receptor protein-tyrosine kinases on endothelial cells and BMDCs, namely VEGFR1 (i.e. Flt-1), VEGFR2 (i.e. FLK-1/KDR) and VEGFR3 (Flt-4) as well as with two non-enzymatic co-receptors, neuropilin-1 and -2 (NP-1 and NP-2), which are expressed on vascular endothelium and neurons. In addition, several of the VEGF family members bind heparan sulfate proteoglycans that are found on the plasma membrane and in the extracellular matrix [32, 33]. The different VEGF members possess distinct binding specificities to the separate receptors, which may contribute to their divergent physiological functions (Table 3.4).

#### *3.3.2.1 VEGF-A*

VEGF-A (initially referred to as VEGF) is the first identified and best characterized member of the VEGF family. The VEGF-A gene is located on the short arm of chromosome 6 and contains eight exons separated by seven introns [34]. Alternative splicing results in the generation of four major isoforms of which VEGF-$A_{165}$ (i.e. the mature protein of 165 amino acids) is the predominant one, followed by VEGF-$A_{189}$, VEGF-$A_{121}$ and VEGF-$A_{206}$. Other isoforms are less commonly expressed *in vivo* and include VEGF-$A_{183}$, VEGF-$A_{165b}$, VEGF-$A_{162}$, VEGF-$A_{148}$ and VEGF-$A_{145}$ [33, 35, 36]. VEGF-A exerts its angiogenic effects through interaction with VEGFR-1, VEGFR-2, NP-1 and NP-2. VEGFR-2 is the main receptor for mediating the angiogenic effects of VEGF-A, whereas VEGFR-1 has been proposed as a ‘decoy’ receptor [37, 38]. The expression of VEGF-A is directly upregulated by HIF-1 under hypoxic conditions [39]. Furthermore, several major growth factors, including PDGF, EGF, TGF-α/-β and bFGF as well as inflammatory mediators like $PGE_2$, TNF-α, IL-1α and IL-6 upregulate the expression of VEGF-A [35, 40].

| Ligand | Isoforms | Receptor | Solubility | Source in adults | Biological activities | Phenotype of knockout mouse |
|---|---|---|---|---|---|---|
| VEGF-A | VEGF-$A_{121}$, VEGF-$A_{165}$, VEGF-$A_{189}$, VEGF-$A_{206}$ (VEGF-$A_{183/145/148/162/165b}$ have also been described) | VEGFR-1 and VEGFR-2; VEGF-$A_{165}$ binds to neuropilin-1 and -2; VEGF145 to neuropilin-2 | VEGF-$A_{121}$ soluble, longer forms bind to heparan sulfates with increasing affinity | Almost all vascularized tissues, especially fenestrated and sinusoidal endothelium | Vasculogenesis, angiogenesis, vascular homeostasis, vascular permeability and recruitment of BMDCs | Loss of single VEGF allele leads to embryonic lethality due to impaired vasculogenesis and angiogenesis |
| VEGF-B | VEGF-$B_{167}$ and VEGF-$B_{186}$ | VEGFR-1 and neuropilin-1 | VEGF-$B_{167}$ binds to heparan sulfates; VEGF-$B_{186}$ soluble | Heart, skeletal muscle and vascular smooth muscle cells | Survival factor, recruitment of BMDCs | Almost-normal phenotype with minor defects (impaired recovery from ischemia, reduced heart size, prolonged PQ-time) |
| VEGF-C | Unprocessed and proteolytically processed (DNDC) forms | VEGFR-2, VEGFR-3 and neuropilin-2 | Soluble | Neuroendocrine organs, lung, heart, kidney and vascular smooth muscle cells | Development of lymphatics and lymphangiogenesis; angiogenesis | Lethal due to impaired development of lymphatics |
| VEGF-D | Unprocessed and proteolytically processed (DNDC) forms | VEGFR-2, VEGFR-3 and neuropilin-2 (heparan-sulfate-dependent) | Soluble | Neuroendocrine organs, lung, heart, skeletal muscle, intestine and vascular smooth muscle cells | Lymphangiogenesis and angiogenesis | Normal |
| PlGF | $PlGF_{131}$ (PlGF-1), $PlGF_{152}$ (PlGF-2), $PlGF_{203}$ (PlGF-3) $PlGF_{224}$ (PlGF-4) | VEGFR-1; PlGF1-2 binds to neuropilin-1 and -2 | PlGF-1 and PlGF-3 soluble; PlGF-2 binds to heparan sulfates | Placenta, thyroid and lung | Angiogenesis, monocyte migration, BMDC recruitment, up-regulation of VEGF-A | Almost-normal phenotype and fertile with minor defects in vascular growth in pathological conditions |

**Table 3.4 - The human VEGF family of cytokines and receptors.** Adapted from Hoff et al. [20]

VEGF-A is the most effective and pleiotropic angiogenic factor, and induces proliferation, sprouting, survival and tube formation of endothelial cells [35, 41, 42]. In addition, VEGF-A also causes vasodilation and hyperpermeability of the endothelium [43, 44]. Besides its role on endothelial cells, VEGF-A induces mobilization of hematopoietic stem cells from the bone marrow, monocyte chemoattraction and osteoblast-mediated bone formation [35]. The vital role of VEGF-A in physiologic vasculogenesis is demonstrated in VEGF-A null mice, which die at embryonic day 8.5 due to impaired vasculogenesis. Also the loss of a single VEGF-A allele leads to embryonic death (between days 11 and 12) due to vascular deformities [45, 46]. Besides embryonic vasculogenesis, VEGF-A affects physiological angiogenic processes during wound healing, ovulation, maintenance of blood pressure, menstruation and pregnancy [35, 36]. Moreover, established vascular beds in the adult intestine, pancreas, thyroid and liver depend on VEGF-A for their maintenance and to prevent partial regression [47, 48]. Overexpression of VEGF-A in the skin causes abundant cutaneous angiogenesis and accelerates experimental tumor growth in transgenic mice, whereas loss of VEGF-A delays wound healing and impedes tumor formation [49-51]. VEGF-A is a key effector also in pathological conditions in which angiogenesis is involved, including cancer as well as ocular, inflammatory, vascular and ischemic diseases [40].

As described above, VEGF-A plays a critical role in all mechanisms of tumor vasculogenesis (Table 3.3). In line herewith, VEGF-A is expressed in practically all solid tumors and some hematological malignancies, including melanoma, glioblastoma, colorectal, gastric, lung, and breast cancer [52]. In addition, high levels of VEGF-A have been associated with disease progression and reduced survival in several cancers like lung, colorectal, gastric and breast cancer [53-56]. The critical involvement of VEGF-A in tumor vasculogenesis has led to the development of anti-angiogenesis therapy against VEGF-A, which will be further discussed in section 3.4.2.1.

#### *3.3.2.2 VEGF-B*

The VEGF-B gene is located on chromosome 11 and contains eight exons. Alternative splicing yields two isoforms, VEGF-$B_{167}$ and VEGF-$B_{186}$ [57, 58]. VEGF-$B_{167}$ is the predominant form and binds to heparan sulfate proteoglycan whereas VEGF-$B_{186}$ is secreted and freely diffusible [33]. The VEGF homology domain of VEGF-B is approximately 47% and 37% identical in

amino acid sequence to those of VEGF and PlGF respectively [59]. The promoter region of VEGF-B contains transcription factor binding sites for Sp1 and AP-2, just like VEGF-A. However, in contrast to VEGF-A, the VEGF-B promoter lacks HIF-1 and AP-1 sites, rendering VEGF-B expression insensitive to hypoxia or cold [60, 61]. Similarly, the expression of VEGF-B is not induced by molecular stimulators of VEGF-A expression like growth factors or steroid hormones [61, 62]. Whatever molecular mechanism regulates the expression of VEGF-B remains poorly understood [59, 63].

The *in vivo* role of VEGF-B has long been elusive since VEGF-B appeared to be inert without an obvious function under normal conditions [59, 63]. VEGF-B deficient mice are largely healthy and also their pathological angiogenesis is not affected in the organs studied, such as wounded skin and hypoxic lung or limb [59, 64, 65]. In addition, VEGF-B is the only VEGF family member whose transgenic overexpression did not induce angiogenesis or lymphangiogenesis [65]. However, instead of an angiogenic factor, VEGF-B has recently been shown to act as a survival factor for vascular endothelial and mural cells as well as their progenitor cells [29]. VEGF-B appears to be critical for the survival of blood vessels under pathological or stress conditions [59]. The survival effects of VEGF-B are established by promoting the expression of vascular pro-survival genes and inhibiting the expression of pro-apoptotic genes via VEGFR-1 and NP1 [29, 66]. In addition to its survival effect on blood vessels, VEGF-B is also a survival factor for different types of neurons, including brain cortical neurons, retinal neurons and motor neurons in the spinal cord [66, 67]. In contrast, recent studies indicate a highly unexpected role of VEGF-B as an endogenous inhibitor of growth and angiogenesis under certain conditions [68]. Apparently, the specific context determines if VEGF-B will act as a survival factor or as an anti-growth/anti-angiogenesis factor (Figure 3.4). Under degenerative conditions, VEGF-B exerts a survival function to protect from cell death. VEGF-B acts instead as a suppressive factor to prevent the overgrowth of tumors, blood vessels or body mass in the presence of high levels of growth factors [59]. Thus, the role of VEGF-B appears to be safeguarding the homeostatic balance between blood vessel growth and blood vessel degeneration to ensure normal blood vessel density and integrity [59].

VEGF-B expression has been demonstrated in a broad range of human cancers [69]. Increased VEGF-B expression levels have been observed in ovarian [70], colorectal [71], renal-cell [72] and prostate cancer [73]. In addition, the expression of VEGF-B correlates with disease stage in

neuroblastoma [74], and with advanced stage, tumor multiplicity and vascular invasion in hepatocellular carcinoma [75].

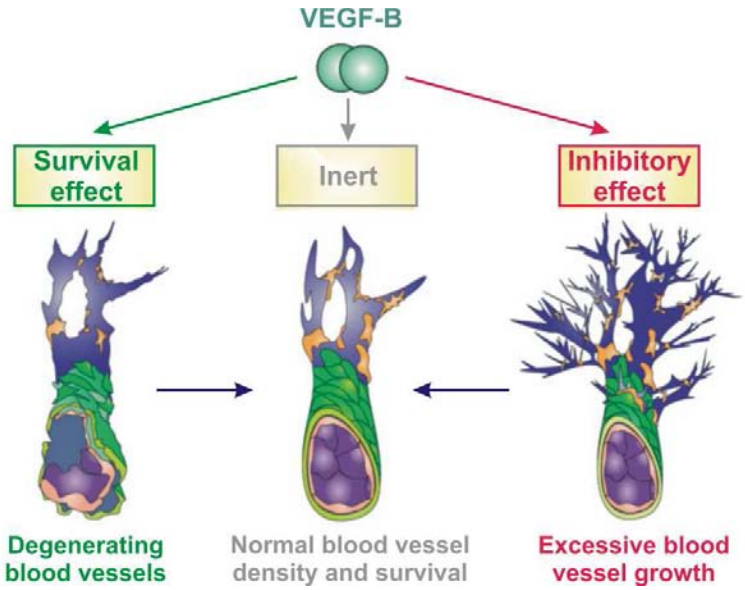


**Figure 3.4 - Multifaceted actions of VEGF-B under different conditions.** ***Left***: under degenerative conditions VEGF-B acts as a survival factor for different types of vascular cells to rescue the endangered blood vessels from degeneration. ***Middle***: under normal conditions VEGFB displays no obvious function and appears to be inert. ***Right***: in the presence of high levels of potent angiogenic/growth factors, VEGF-B may act as an inhibitory factor to prevent excessive blood vessel or tissue growth. Figure from Li et al. [59].

### *3.3.2.3 VEGF-C*

The VEGF-C gene is located on chromosome 4 and is made up of seven exons but does not undergo alternative splicing [58]. The synthesized precursor VEGF-C protein goes through an intricate proteolytic maturation that generates multiple processed forms which bind and activate VEGFR-3 and NP-2. Only the fully mature VEGF-C protein can also bind VEGFR-2 [33, 36, 76]. The promoter region of VEGF-C contains binding sites for Sp-1, AP-2 and NF-κB transcription factors, which implies the regulation of VEGF-C expression by PKA and TGF-β (AP-2) as well as by IL-1 and TNF-α (NF-κB) [77]. Although an hypoxia responsive element (HRE) is absent, hypoxia has been shown to induce VEGF-C expression [78, 79]. In addition, VEGF-C is upregulated by $PGE_2$ [80, 81].

VEGF-C induces proliferation, migration and survival of endothelial cells and is involved in developmental lymphangiogenesis as well as the maintenance of adult lymphatic vasculature [82]. The vital role of VEGF-C in the development of lymphangiogenesis is indicated by the embryonic lethality of VEGF-C null mice [83]. On the other hand, VEGF-C is not involved in

developmental angiogenesis because blood vessels are normal in VEGF-C null mice [83]. Heterozygous loss of VEGF-C results in a defective lymphatic development and causes lymphedema [83]. Adenoviral VEGF-C gene transduction induces the growth of lymphatic vessels in several different animal models [84, 85].

The expression of VEGF-C has been observed in a significant fraction of human cancers including breast [86], cervix [87], colon [88], lung [89], prostate [90] and stomach cancer [91]. VEGF-C plays an important role in tumor lymphangiogenesis and in the formation of lymph node metastases [92-94]. Moreover, several clinical studies in cancer patients have shown a positive correlation between the expression of VEGF-C and lymphatic invasion and metastasis as well as patient survival [90, 91, 93, 95].

#### *3.3.2.4 VEGF-D*

The VEGF-D (or c-fos induced growth factor (FIGF)) gene is located on the X chromosome and contains seven exons [96]. Like VEGF-C, VEGF-D does not undergo alternative splicing but is synthesized as precursor protein. Proteolytic processing generates the mature form of VEGF-D which binds to both VEGFR-2 and VEGFR-3 [97]. In addition, VEGF-D binds NP-2 in a heparan sulfate-dependent manner [76]. The VEGF-D promoter contains a canonical AP-1 binding site [96]. The expression of VEGF-D is also regulated by hypoxia though no HRE was found in the promoter region [79, 98].

VEGF-D promotes the growth of blood and lymphatic vessels by induction of endothelial cell proliferation, migration, and survival [99]. However, VEGF-D null mice are viable and exhibit a normal lymphatic vasculature, suggesting that other growth factors can substitute for VEGF-D [100].

VEGF-D enhances lymphangiogenesis, angiogenesis and metastatic spread in experimental tumors [101]. In humans, the expression of VEGF-D is upregulated in breast [102], cervical [103], ovarian [104] and thyroid cancer [105] as well as in glioblastoma [106]. In addition, the expression of VEGF-D correlates with metastatic spread and poor outcome in melanoma [107], gastric [108], colorectal [109, 110], lung [111], and ovarian carcinomas [112]. However, other studies reported contradictory results with reduced VEGF-D expression in colorectal cancer [88] or an inverse correlation of VEGF-D expression with prognostic indicators in lung [113] and colorectal cancer [114].

### *3.3.2.5 PlGF*

The human PlGF gene is mapped on chromosome 14 and contains seven exons [115]. Alternative splicing yields four isoforms, namely PlGF-1 ($PlGF_{131}$), PlGF-2 ($PlGF_{152}$), PlGF-3 ($PlGF_{203}$), and PlGF-4 ($PlGF_{224}$), which all bind to VEGFR-1 [116-118]. PlGF-2 can also bind to both NP-1 and NP-2 [119]. The promoter region of PlGF contains recognition sequences for metal transcription factor 1 (MTF-1) and NF-κB, and both have been shown to modulate PlGF expression under hypoxic conditions [120, 121]. Although there is no HRE in the PlGF promoter region, the expression of PlGF is induced by hypoxia and stimulated by HIF-1α [122, 123].

PlGF is a pleiotropic factor regulating various biological activities and affecting multiple cell types. It exerts survival, migration, proliferation, metabolism and activation effects on vascular (i.e. endothelial and mural cells) as well as nonvascular cells (e.g. macrophages, BM-derived progenitors, fibroblasts, dendritic cells and neurons) [5]. Yet, PlGF is dispensable for development and physiolgical homeostasis in adults [124]. The redundancy of PlGF in health is demonstrated by PlGF knockout mice which do not exhibit an apparent phenotype [124]. However, these mice recover poorly after myocardial infarction. So, PlGF is important in pathological conditions where loss of PlGF impairs angiogenesis, collateral vessel growth during ischemia, wound healing, inflammation and cancer [124]. In line herewith, up-regulation of PlGF has been found in several conditions associated with pathologic angiogenesis like ischemic myocardial infarction [125], cerebral and limb ischemia [126, 127], as well as skin wound healing [128], sepsis [129], atherosclerosis [130], rheumatoid arthritis [131] and colitis [132].

Also in several cancers, the expression of PlGF is increased. In addition, PlGF levels correlate with tumor stage, invasion, metastasis, tumor recurrence and inversely with survival in several cancers, including those of the breast[133], stomach [134], lung [135] and colon [136]. PlGF may promote tumor growth via various mechanisms and cell types, summarized in figure 3.5. In addition, PlGF is associated with relapse of cancer patients treated with VEGF-A inhibitors [137]. The pleiotropic effects of PlGF on tumor angiogenesis has led to the development of an antibody against PlGF for the treatment of cancer. This antibody is further discussed in section 3.4.2.1.

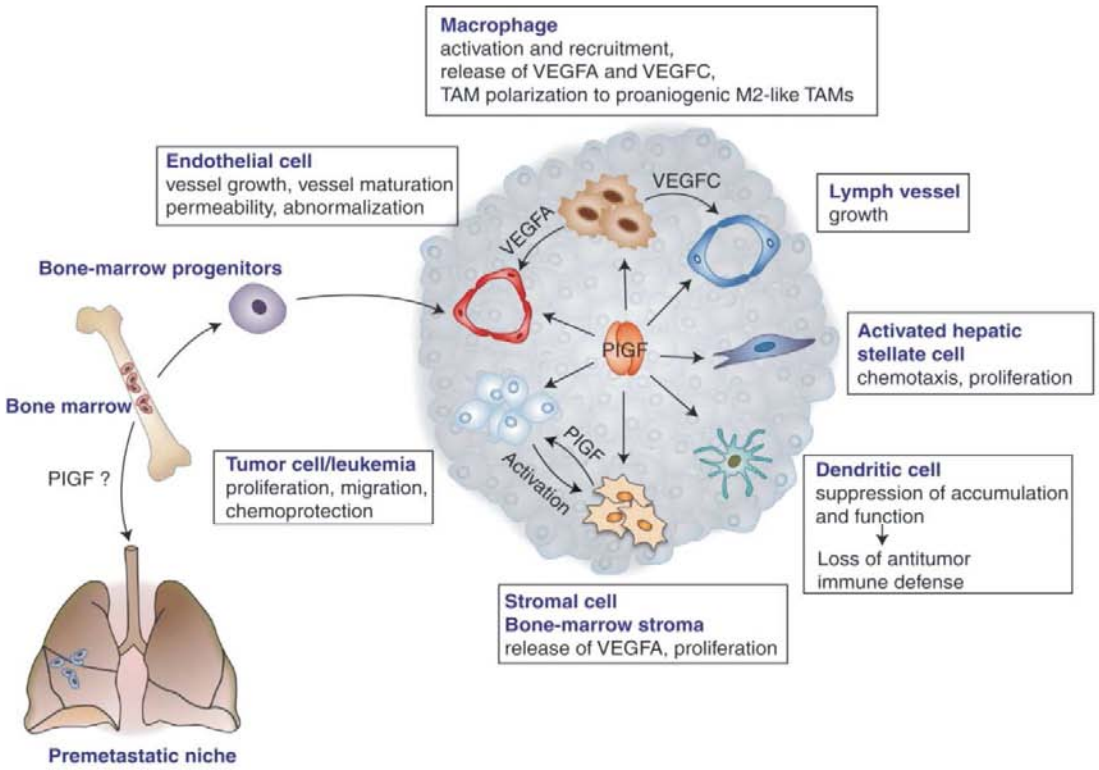


**Figure 3.5 - Pleiotropic effects of PlGF on multiple cell types in cancer.** In a tumor, PlGF affects multiple cell types and processes. PlGF promotes angiogenesis by promoting proliferation and migration of endothelial cells, maturation of the vessels and mobilization of BM progenitors. The recruitment of macrophages provides additional angiogenic and lymphangiogenic factors. To suppress anti-tumor immune responses, PlGF reduces the accumulation and function of dendritic cells. PlGF also stimulates proliferation of tumor cells which in turn activate stromal cells to produce PlGF. In hepatocellular carcinoma, PlGF stimulates the proliferation and migration of activated hepatic stellate cells. TAM: tumor-associated macrophage. PlGF may also be implicated in the mobilization of BM-derived progenitor cells to pre-metastatic niches. Figure from Dewerchin et al. [5]

## 3.4 Anti-angiogenesis therapy

### 3.4.1 Introduction

Considering the dependence of a solid tumor on angiogenesis, therapies targeting tumor vascularization to starve the tumor to death appear highly promising. Such strategy was first proposed by Judah Folkman in 1971 [138]. New insights on the complex nature of angiogenesis along with the ongoing identification of pro- and anti-angiogenic factors, have provided several potential targets for the development of anti-angiogenesis therapies. However, except for drugs targeting VEGF ligands and receptors, most of the angiogenesis inhibitors failed to fulfill the expectations raised by promising preclinical results [139]. In addition, the concept of “starving” tumors by obliterating their blood supply has been revoked because aggressive angiogenesis

inhibition may aggravate tumor metabolism and/or promote metastasis [140, 141]. The current goal for anti-angiogenic therapy therefore is to correct the abnormal structure and functionality of tumor vessels, making them more accessible for the delivery of chemotherapeutic drugs [142]. Several anti-angiogenic therapies are currently approved for the treatment of various cancers and other therapies are in late stage-clinical development [20]. These are summarized in Table 3.5.

| **Therapeutic agent/trade name** | **VEGF family target(s)** | **Target(s) other(s)** | **Clinical development (main indications)** | **References** |
|---|---|---|---|---|
| Monoclonal antibodies | | | | |
| Bevacizumab/Avastin (Genentech Inc.) | VEGF-A | - | Approved for CRC, RCC, NSCLC, GB, | [143] |
| Cetuximab/Erbitux (Bristol-Myers Squibb/Eli Lilly and Company) | - | EGFR | Approved for CRC, H&NC | [144] |
| Panitumumab/Vectibix (Amgen) | - | EGFR | Approved for CRC | [145] |
| Nimotuzumab/Theraloc (Oncoscience) | - | EGFR | Phase III (GB, H&NC) | [146, 147] |
| Zalutumumab/HuMax-EGFr (Genmab) | - | EGFR | Phase III (H&NC) | [148, 149] |
| Necitumumab/IMC-11F8 (Bristol-Myers Squibb/Eli Lilly and Company) | - | EGFR | Phase III (NSCLC, CRC) | [150, 151] |
| Ramucirumab/IMC-1121B (ImClone Systems) | VEGFR-2 | - | Phase III (BC, HCC, NSCLC, GC, CRC) | [152] |
| IMC-18F1/ Icrucumab (ImClone Systems) | VEGFR-1 | - | Phase II | [153] |
| mabPlGF/TB-403 (Thrombogenics) | PlGF | - | Phase I | [154] |
| Tyrosine kinase inhibitors | | | | |
| Sorafenib/Nexavar (Bayer/Schering.) | VEGFR-1, -2, -3 | PDGFR, Raf, Flt3, c-Kit | Approved for RC, HCC | [155] |
| Sunitinib/Sutent (Pfizer) | VEGFR-1, -2, -3 | PDGFR, c-Kit, Flt3, RET, CSF-1R | Approved for RCC, HCC, GIST, PNET | [156] |
| Pazopanib/Votrient (GlaxoSmithKline) | VEGFR-1, -2, -3 | PDGFR, c-Kit, Itk, Lck, c-Fms | Approved for RCC | [157] |
| Gefitinib/Iressa (AstraZeneca/Teva) | - | EGFR | Approved for NSCLC | [158] |
| Erlotinib/Tarceva (Genentech Inc.) | - | EGFR | Approved for NSCLC, PaC | [159] |
| Vandetanib/Caprelsa (AstraZeneca) | VEGFR-2 | EGFR, RET | Approved for MTC | [160] |
| Lapatinib/Tykerb (GlaxoSmithKline) | - | EGFR, HER-2 | Approved for BC | [161] |

| Therapeutic agent/trade name | VEGF family target(s) | Target(s) other(s) | Clinical development (main indications) | References |
|---|---|---|---|---|
| Tyrosine kinase inhibitors (continuation) | | | | |
| Regorafenib/Stivarga (Bayer) | VEGFR-2, -3 | PDGFR, Raf, RET, c-Kit, FGFR | Approved for CRC | [162] |
| Cabozantinib/Cometriq (Exelixis) | VEGFR-2 | c-Met, RET, c-Kit, Flt3 | Approved for MTC | [163] |
| Vatalanib/ PTK787 (Novartis Pharmaceuticals) | VEGFR-1, -2 | PDGFR, c-Kit | Phase III (CRC) | [164] |
| Neratinib/ HKI-272 (Puma Biotechnology Inc.) | - | EGFR, HER-2 | Phase III (BC) | [165] |
| Brivanib alaninate (Bristol-Myers Squibb) | VEGFR-2 | FGFR | Phase III (HCC, CRC) | [166] |
| Cediranib/Resentin (AstraZeneca) | VEGFR-1, -2, -3 | - | Phase III (CRC, GB, OC, BTC, NSCLC) | [167-169] |
| Icotinib (BetaPharma) | - | EGFR | Phase III (NSCLC) | [170] |
| Afatinib/Tomtovok (Boehringer Ingelheim) | - | EGFR, HER-2 | Phase III (NSCLC, H&NC, BC) | [171, 172] |
| Axitinib/Inlyta (Pfizer) | VEGFR-1, -2, -3 | PDGFR, c-Kit | Phase III (RCC, PaC) | [173, 174] |
| Nintedanib/Vargatef (Boehringer Ingelheim) | VEGFR-2 | PDGFR, FGFR | Phase III (NSCLC, OC, BTC) | [175] |
| Linifanib (Abott) | VEGFR-1, -2, -3 | PDGFR | Phase II (HCC ) | [176] |
| Motesanib (Amgen) | VEGFR-1, -2, -3 | PDGFR, c-Kit | Phase III (NSCLC) | [177] |
| Tivozanib (Abott) | VEGFR-1, -2, -3 | - | Phase III (RCC) | [178] |
| Dovitinib (Novartis Pharmaceuticals) | VEGFR-1, -2, -3 | FGFR | Phase III (RCC) | [179] |
| Receptor fusion protein | | | | |
| Aflibercept/Zaltrap (Regeneron/Sanofi-Aventis) | VEGF-A, VEGF-B, PlGF | - | Approved for CRC | [180] |

c-Fms: transmembrane glycoprotein receptor tyrosine kinase, c-Kit: v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog, c-Met: hepatocyte growth factor receptor, CSF-1R: colony stimulating factor 1 receptor, EGFR: epidermal growth factor receptor, FGFR: fibroblast growth factor receptor, Flt-3: fms-like tyrosine kinase 3, HER-2: human epidermal growth factor receptor 2, Itk: interleukin-2 receptor inducible T-cell kinase, Lck: leukocyte-specific protein tyrosine kinase, PDGFR: platelet-derived growth factor receptor, PlGF: placental growth factor, Raf: murine leukemia viral oncogene, RET: ret proto-oncogene

BC: breast cancer, BTC: biliary tract cancer, CRC: colorectal cancer, GB: glioblastoma, GC: gastric adenocarcinoma, GIST: gastrointestinal stromal tumor, H&NC: head and neck cancer, HCC: hepatocellular carcinoma, MTC: medullary thyroid cancer, NSCLC: non-small cell lung cancer, OC: ovarian cancer, PaC: pancreatic cancer, PNET: pancreatic neuroendocrine tumors, PrC: prostate cancer, RCC: renal cell carcinoma

**Table 3.5 - Approved and late-stage development therapeutic agents that target angiogenesis in the treatment of cancer**. Adapted from Hoff et al. [20] and Tugues et al. [181].

### 3.4.2 VEGF family-targeting therapy

Because the VEGF family and their receptors are crucial mediators involved in every mechanism of tumor vascularization, it is not surprising that many therapeutic agents have been developed that target in one way or the other these mediators of tumor angiogenesis. These therapeutic agents are either monoclonal antibodies or tyrosine kinase inhibitors (Table 3.5, Figure 3.6).

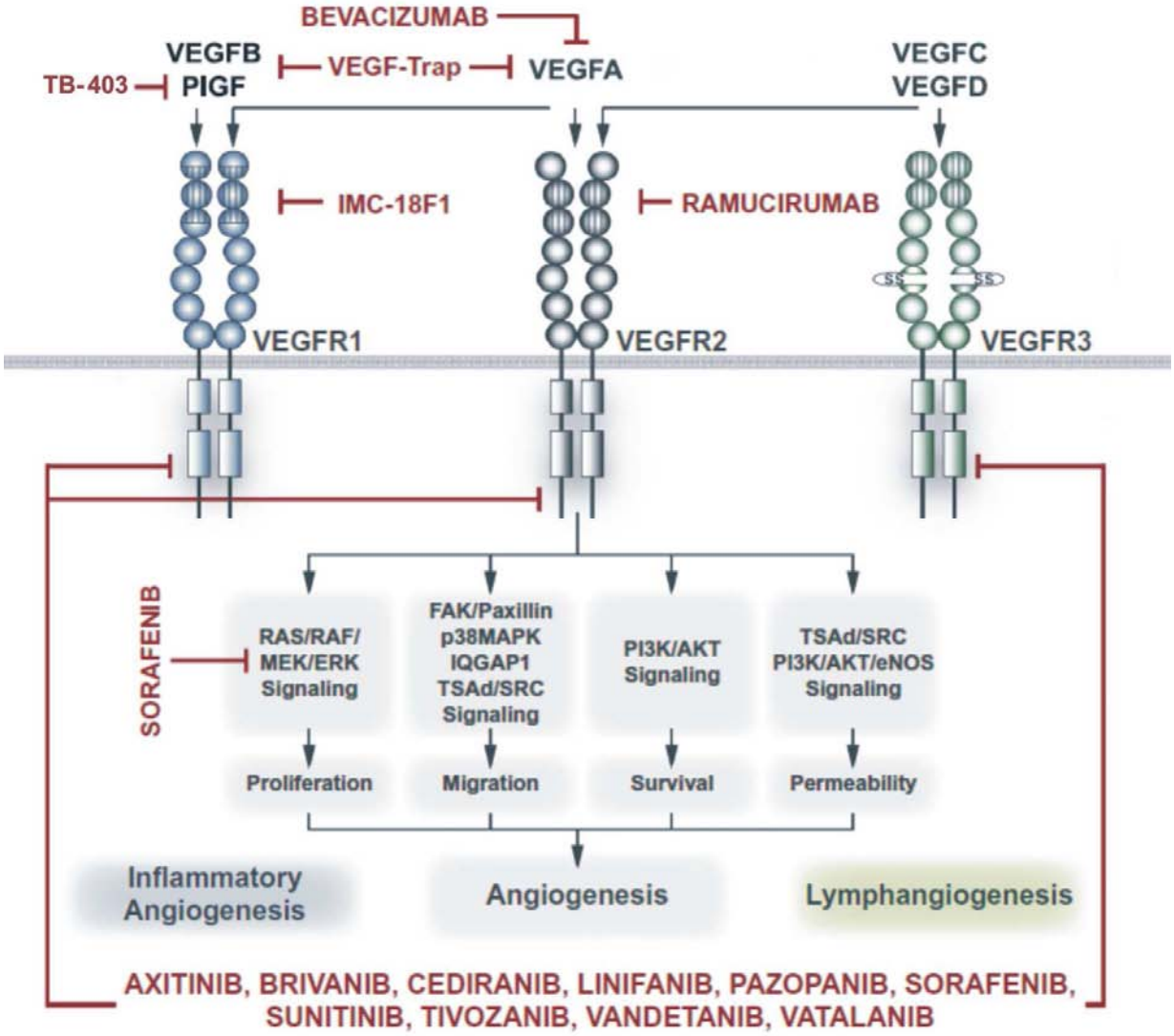


**Figure 3.6 - VEGF signaling inhibitors and their targets.** The human VEGF family members (VEGF-A, -B, -C, -D and PlGF) bind to their cognate receptors (VEGFR1, VEGFR2 and VEGFR3) as indicated. VEGF antagonists either interfere with binding of VEGF ligands on the extracellular domain of receptors (Bevacizumab, VEGF-Trap, Veglin, IMC-18F1, Ramuciumab/CDP791) or compete for ATP-binding to the intracellular kinase domain (axitinib, brivanib, cediranib, linifanib, pazopanib, sorafenib, sunitinib, tivozanib, vandetanib, vatalanib). Figure adapted from Tugues et al. [181]

#### *3.4.2.1 Monoclonal antibodies*

Monoclonal antibodies bind either a VEGF ligand or a receptor for VEGF and consequently inhibit the interaction between ligand(s) and receptor(s) (Figure 3.6).

**Bevacizumab** is a humanized monoclonal antibody against VEGF-A and the first approved anti-angiogenic agent for the treatment of cancer. In preclinical xenograft models, tumor growth and metastasis were inhibited by bevacizumab as single-agent therapy. Combination of bevacizumab with chemotherapy or radiotherapy, resulted in a synergistic inhibition of tumor growth with decreased vascular permeability and tumor vessel diameter and density [182]. Several mechanisms are postulated for the precise mechanism by which bevacizumab enhances chemotherapy. These mechanisms include: normalization of the tumor vasculature causing improved delivery of cytotoxic agents [183]; direct effect on cells that are dependent on VEGF-A as a growth and survival factor [184]; sensitization of tumor endothelial or circulating cells to cellular damage [185]. Clinical phase I trials have found bevacizumab to be relatively well-tolerated and non-toxic [186]. The addition of bevacizumab to IFL chemotherapy (i.e. ironotecan, 5-FU and leucovorin) significantly increased the median duration of survival of metastatic colorectal cancer patient with 4.7 months as compared to IFL alone [187]. These overall positive results have led to the approval of bevacizumab by the FDA and EMEA [188]. Since its approval in 2004, bevacizumab is used as first-line treatment for metastatic colorectal cancer in combination with established chemotherapeutic agents, including 5-FU, capecitabine, irinotecan and oxaliplatin, as well as combinations of these agents [188-190]. The ECOG E3200[1] study found that the addition of bevacizumab to FOLFOX4 (i.e. oxaliplatin, 5-FU and leucovorin) significantly increased the overall survival and progression-free survival by 2.1 and 2.6 months respectively, as compared to FOLFOX4 alone in previously treated metastatic colorectal cancer patients [191]. These results led to the approval in 2006 of bevacizumab also as second-line treatment for metastatic colorectal cancer [32, 188]. In addition to its usage as first- and second-line treatment of metastatic colorectal cancer, the use of bevacizumab has expanded to non-small cell lung cancer, glioblastoma and metastatic renal cell carcinoma [143].

**Ramucirumab** is a fully human monoclonal antibody that binds VEGFR-2, thus blocking VEGF-A from binding. As such, preclinical studies showed that ramucirumab inhibits cell

[1] Eastern Cooperative Oncology Group E3200

proliferation *in vitro* as well as tumor progression in murine xenograft models [152, 192]. Ramucirumab was well tolerated in phase I clinical trials and these studies also showed promising levels of partial responses and stable disease [193]. Therefore, ramucirumab is currently being evaluated in about 30 clinical studies, including phase III clinical trials for colorectal cancer, gliomas, head and neck cancer as well as breast, gastric and non-small cell lung cancer (Table 3.5) [194].

**IMC-18F1** is a fully human monoclonal antibody that targets the human VEGFR-1, thereby blocking the binding of VEGF-A, VEGF-B, and PIGF to the receptor. Based on preclinical studies, IMC-18F1 may provide clinical benefit to cancer patients as it suppressed tumor growth in xenograft models [153, 195]. Phase I clinical trials have indicated a favorable safety profile for IMC-18F1 mono-therapy [196]. IMC-18F1 is currently evaluated in phase II clinical trials for the treatment of advanced colorectal, breast and urologic cancers [197-199].

**TB-403** is a humanized recombinant monoclonal antibody directed against PlGF. By binding to PlGF, TB-403 inhibits the interaction of PlGF with VEGFR-1, which may result in the inhibition of tumor angiogenesis and tumor cell proliferation. TB-403 inhibits growth and metastasis in various tumor models and exhibits an exceptional safety profile in preclinical *in vivo* models compared with other anti-angiogenic treatments [200]. Based on the favorable safety profile, which was confirmed in phase I clinical studies, and supportive preclinical proof-of-concept studies, further clinical studies with TB-403 are now in progress [201, 202].

#### *3.4.2.2 Tyrosine kinase inhibitors*

Tyrosine kinase inhibitors are small molecular weight compounds that block VEGFR2 signaling by competing for the ATP-binding site in the kinase. Often, these inhibitors target a broad spectrum of kinases due to the high degree of conservation of the ATP-binding sites of tyrosine kinases [203]. Nevertheless, tyrosine kinase inhibitors differ from each other in the spectrum of targeted kinases, their pharmacokinetics as well as specific adverse effects [204]. Although tyrosine kinase inhibitors are overall well tolerated, they are also associated with an increased risk of developing potentially life-threatening conditions such as arterial thrombotic events, bleeding and congestive heart failure [204, 205]. Selective tyrosine kinase inhibitors may minimize these adverse effects. Multi-target kinase inhibitors or combination therapy containing these inhibitors may affect multiple angiogenic pathways, thus providing a broader efficacy and

resistance [203]. Currently, six tyrosine kinase inhibitors targeting VEGFR(s) are approved for use in specific cancers, namely sorafenib, sunitinib, pazopanib, vandetanib, regorafenib and cabozantinib [155-157, 160, 162, 163]. Many others are in late-stage clinical development (Table 3.5). Preclinical and clinical results of these inhibitors are reviewed by Ribatti [206] and specific for advanced colorectal cancer by Grothye and Allegra [188].

#### *3.4.2.3 VEGF-A Trap*

Aflibercept also known as VEGF-Trap, is a recombinant, decoy receptor fusion protein that binds VEGF-A with higher affinity than its native receptors. In addition, it prevents also VEGF-B and PlGF from binding to their receptors [207, 208]. For this purpose, it comprises the second immunoglobulin (Ig) domain of VEGFR-1 and the third Ig domain of VEGFR-2, fused to the constant region of human IgG1 [209]. In different tumor mouse models, aflibercept significantly reduces tumor growth and vascularization and extends survival [209, 210]. These preclinical results have supported the investigation of aflibercept in clinical trials. Phase I and II clinical trials have revealed a significant survival advantage and patient-dependent clinically meaningful results, as well as a manageable safety profile [210]. Aflibercept is currently in phase III clinical trials for the treatment of prostate, ovarian and non-small cell lung cancer and has been approved for the treatment of advanced colorectal cancer in 2012 (Table 3.5).

### 3.4.3 Resistance to VEGF therapy

Despite clinical benefits such as improved 2-year survival in patients treated with drugs targeting the VEGF pathway, the global impact of the current agents is relatively modest as no gains have been made in 5-year survival [211]. VEGF targeting agents may lead to disease stabilization and prolonged periods of progression free or overall survival in patients with metastatic disease, but eventually tumors become non-responsive or do not respond at all. As a consequence, the benefits in progression free or overall survival time are mostly measured in months [140].

A tumor may evade specific anti-angiogenic therapies by the activation of compensatory mechanisms involving alternative pro-angiogenic signaling, resulting in intrinsic or adaptive resistance. Signaling by redundant angiogenic pathways are a major mechanism of this resistance [212]. Alternative pathways in human non-cancerous cells may help to overcome deviations in normal signaling, but in cancer cells the same pathways may offer resistance to current anti-VEGF therapies. In line herewith, treatment of pancreatic tumors with anti-VEGFR agents has

been demonstrated to increase the expression levels of among others members of the FGF family and angiopoietins [213]. Increased levels of these factors may allow persisting neovascularization despite VEGF-A blockade. Also increased levels of non-tumor-derived pro-angiogenic factors may contribute to resistance. Systemic increases in proangiogenic factors like VEGF-A, PlGF, G-CSF, bFGF and SDF-1 have been observed after administration of VEGF(R) inhibitors and in colon cancer recurrence [214, 215]. The increased systemic levels of these factors may provide alternative pathways for angiogenesis as well as engulf anti-VEGF therapy, causing resistance. In addition, these factors may also facilitate secondary metastasis [216]. Other major mechanisms of resistance derives from the selection of hypoxia-resistant tumor cells by anti-angiogenic drugs and recruitment of myeloid and circulating cells for VEGF-independent angiogenesis [140, 217]. Approaches to overcome this resistance may include the combined blocking of redundant angiogenic pathways and ligands, inhibition of cellular migration and the use of vascular disruptive agents such as tubulin-binding agents, flavonoids and TNF [216, 218]. Clearly, such approaches will require an individualized approach based on biomarkers and/or gene expression profiling for the selection of responders to a specific therapy. Wild-type KRAS is a predictive marker for EGFR-therapy since activating mutations in KRAS are recognized as a strong predictor of resistance to EGFR-targeted drugs [219]. However, no others biomarkers are yet known to predict reliably whether or not a patient should receive a particular therapy [211]. Continued research to fully clarify cellular and molecular angiogenesis networks and to identify eligible predictive biomarkers will lead us to treat cancer patients with personalized combination treatments exhibiting the highest level of efficacy and safety.

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# Part II

# Objectives and strategy

## Objectives and strategy

The present assessment of the expression levels in colon cancer of the individual VEGF family members still has some major limitations. Despite several studies documenting the (mRNA) overexpression of certain VEGF family members, the data are scattered and often contradictory. As a result it is challenging to obtain a global picture of the expression of the VEGF family in colon cancer. We have assessed two potential mechanisms that may explain why such contradictory and inconsistent results are reported in literature. For a start, several studies have used samples obtained by two different clinical procedures, namely surgical resection or colonoscopic biopsy. We therefore compared in **Chapter 4**, the expression patterns of the VEGF family members between healthy colon and colon carcinoma samples obtained by either biopsy or resection. COX2, 5-LOX, glucose transporter 1 (GLUT-1) and carbonic anhydrase IX (CAIX) were included as markers for cellular stress and hypoxia. This analysis revealed the occurrence of sampling-induced hypoxia in resection samples which influenced the reliability of the VEGF family members as colon cancer markers.

Secondly, published studies have analyzed the VEGF members individually and/or in small groups by means of different analytical methods. In **Chapter 5**, we conducted a systematic and comprehensive study to determine in a single experimental setup the mRNA expression signatures of all VEGF family members in colon carcinoma samples. Comparison with colon adenomas and liver metastases further allowed addressing the mRNA expression of VEGFs during malignant progression. In addition, we determined to what extent individual carcinoma samples concomitantly express VEGF family members, and also the degree to which these individual signatures evolve during progression from adenoma to carcinoma and liver metastasis. These expression signatures were subsequently compared with clinicopathological variables to verify if they could be associated with a specific feature of malignancy. Next we verified if the observed mRNA expression patterns could be translated to serum levels in order to offer a much needed non-invasive and cost-effective screening method for the detection of colon cancer.

Finally, we aimed to clarify the molecular basis of the progression-associated expression patterns of the VEGF family members observed in human colon cancer. Inflammatory eicosanoids derived from COX2 and 5-LOX are pivotal factors in both angiogenesis and tumorigenesis. Because COX2 and 5-LOX have been associated with the expression of VEGF-A and VEGF-C,

we performed a similar systematic expression analysis in **Chapter 6** to determine the mRNA expression levels of COX2 and 5-LOX in relation to the mRNA expression of VEGF family members during malignant progression from adenoma to carcinoma. To verify the causal relation of the observed associations between the expression of eicosanoid enzymes and VEGF family members and the higher risk of malignancy, human colon cell lines were subsequently treated with eicosanoid-stimulatory and -inhibitory conditions in two *in vitro* experiments. In a first setup, we verified the effect of eicosanoids on malignancy with the invasiveness of colon cancer cells as a measurable criteria for malignancy. In a second setup, we verified the impact of COX2- and 5-LOX-derived eicosanoids on the mRNA expression of COX2, 5-LOX and the VEGF family members.

# Part III

# Results

# Chapter 4

# How clinical procedures impact science

**Contributions**

This study was conceived and designed by Sarah Pringels, Nancy Van Damme, Johan Grooten and Marc Peeters.

All technical experiments were performed by Sarah Pringels.

The acquisition of patient data and clinical samples was coordinated by Nancy Van Damme.

Expert advice and support on RT-qPCR expression analysis were provided by Bram De Craene.

## 4.1 Clinical procedure for colon carcinoma tissue sampling directly affects cancer marker-capacity of VEGF family members

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### Abstract

mRNA levels of members of the Vascular Endothelial Growth Factor family (VEGF-A, -B, -C, -D, Placental Growth Factor/PlGF) have been investigated as tissue-based markers of colon cancer. These studies, which used specimens obtained by surgical resection or colonoscopic biopsy, yielded contradictory results. We studied the effect of the sampling method on the marker accuracy of VEGF family members. Comparative RT-qPCR analysis was performed on healthy colon and colon carcinoma samples obtained by biopsy (n=38) or resection (n=39) to measure mRNA expression levels of individual VEGF family members. mRNA levels of genes encoding the eicosanoid enzymes cyclooxygenase 2 (COX2) and 5-lipoxygenase (5-LOX) and of genes encoding the hypoxia markers glucose transporter 1 (GLUT-1) and carbonic anhydrase IX (CAIX) were included as markers for cellular stress and hypoxia. Expression levels of *COX2*, *5-LOX*, *GLUT-1* and *CAIX* revealed the occurrence in healthy colon resection samples of hypoxic cellular stress and a concurrent increment of basal expression levels of VEGF family members. This increment abolished differential expression of *VEGF-B* and *VEGF-C* in matched carcinoma resection samples and created a surgery-induced underexpression of *VEGF-D*. *VEGF-A* and *PlGF* showed strong overexpression in carcinoma samples regardless of the sampling method. Sampling-induced hypoxia in resection samples but not in biopsy samples affects the marker-reliability of VEGF family members. Therefore, biopsy samples provide a more accurate report on VEGF family mRNA levels. Furthermore, this limited expression analysis proposes VEGF-A and PlGF as reliable, sampling procedure insensitive mRNA-markers for molecular diagnosis of colon cancer.

### 4.1.1 Introduction

Colorectal cancer is the second most commonly diagnosed cancer in females and the third in males. It is the second leading cause of cancer-related death [1]. Worldwide, it accounts for over 1.2 million new cases every year, and in 2008 it caused about 608,700 deaths. Colon carcinoma evolves from a premalignant adenoma precursor stage or polyp. The progression from adenoma to carcinoma is a multistep process involving cumulative genetic and epigenetic alterations in proto-oncogenes, tumor suppressor genes and DNA repair genes [2-4].

Colon carcinoma tissue samples have been intensively studied in search for tissue-based diagnostic, prognostic and predictive markers. Samples are routinely obtained by two different clinical procedures. During colonoscopy, which is the gold standard for detection of colon carcinoma and adenoma, biopsies of polyp-like extrusions are obtained for pathological examination, and these extrusions are removed whenever possible. In surgical resection, carcinoma-like outgrowths are removed by cutting out part of the colon containing the suspected outgrowth as well as some surrounding healthy tissue. However, little is known about the impact of the sampling method on the overall condition of the sampled tissue or the expression levels of potential cancer biomarker genes.

Vascular Endothelial Growth Factor (VEGF; VEGF-A) has long been proposed as a biomarker for cancer as well as a target for anti-angiogenic cancer therapy. Several studies consistently showed elevated VEGF-A expression levels in most solid tumors, including colon carcinoma [5-9]. Furthermore, these elevated expression levels have been correlated with tumor progression [10-12]. VEGF-A is an inflammation and hypoxia responsive gene, and its biomarker function is believed to be related to the hypoxic growth conditions characteristically associated with rapidly growing solid tumors and to its ability to promote the development of new vasculature [11, 13].

Fewer studies addressed the mRNA expression levels in colon cancer of the other VEGF family members: VEGF-B, VEGF-C, VEGF-D and Placental Growth Factor (PlGF). Furthermore, some of these studies reported contradictory results. As such, similar expression levels of *VEGF-C* in healthy and carcinoma tissue were reported in three studies [5, 8, 14]. However, other studies reported higher levels [6, 7] that were correlated with lymph node metastasis and poor prognosis [8].

We believe that some of these controversial findings might have resulted from the use of different types of colon tissue samples. Several studies performed expression analysis on samples obtained during surgical resection [5, 7, 8]. Others used biopsies obtained during colonoscopy [14] or did not specify the sampling method [6]. Yet, both sampling procedures differ strikingly; the acquirement of colon biopsies requires only minutes, whereas during surgical resection part of the colon is clamped off for a considerable length of time. To examine to what extent the sampling procedure may affect VEGF gene expression, we analyzed mRNA expression levels of all five VEGF family members in colon carcinoma samples obtained by biopsy and in others obtained by surgical resection. mRNA expression levels in healthy colon tissue of the eicosanoid enzymes, cyclooxygenase 2 (COX2) and 5-lipoxygenase (5-LOX), were included as markers of cellular stress induced by inflammation, tissue damage and/or hypoxia [15-19]. In addition, mRNA expression levels of glucose transporter 1 (GLUT-1) and carbonic anhydrase IX (CAIX) were included as markers of hypoxia [20, 21].

### 4.1.2 Materials and methods

#### Biological samples

Samples were obtained from primary colon carcinomas either by biopsy (n=38) or by surgical resection (n=39) at the Ghent University Hospital. Carcinomas were sampled in the infiltrating area of the growth, avoiding the necrotic center. Histopathological examination confirmed the carcinoma state of the tissue. From each patient, a corresponding healthy colon mucosa sample was taken from the same colon segment. None of the patients had received chemo- or radiotherapy before surgery or colonoscopy. Immediately after isolation, the biopsies were placed in RNA*later*® Solution (Ambion/Applied Biosystems, Foster City, CA). Surgical resection samples were also placed in RNA*later*® Solution at the end of the surgical procedure and after an initial examination by the pathologist. All samples were kept at –80°C until RNA extraction. The clinicopathological features of the patients are summarized in table 4.1. All tissues were obtained following informed consent of the patients and approval of the study by the Ethics Committee of the Ghent University Hospital.

#### RNA extraction, RNA quality control and cDNA synthesis

Total RNA was extracted with the RNeasy Plus mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. This kit contains a gDNA-elimination step to avoid gDNA

contamination. After extraction, RNA quality and integrity was verified using an RNA 6000 Nano Chip Kit on the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Only samples with adequate quality and integrity (77/80) were used for the RT-qPCR analysis. cDNA was synthesized from 1 µg of total RNA using Superscript® II reverse transcriptase (Invitrogen, Merelbeke, Belgium) according to the manufacturer's instructions.

**RT-qPCR**

Real-time quantitative PCR (RT-qPCR) was performed using the LC 480 Sybr Green I master kit on a LightCycler® 480 Real-Time PCR system (both from Roche Applied Science, Penzberg, Germany). Primers were designed using PrimerSelect (DNASTAR, Madison, USA) and purchased from Invitrogen. The primers were designed for gene-specific expression profiling and cover all splice variants. The sequences of the forward and reverse primers were as follows: VEGF-A 5'-TGAGTTGCCCAGGAGACCAC-3' and 5'-GAAGGGGAGCAGGAAGAGGAT-3'; VEGF-B 5'-CCGGAAGCTGCGAAGGTGACA-3' and 5'-GGGAGACAAGGGATGGCAGAAGAG-3'; VEGF-C 5'-CACGGCTTATGCAAGCAAAGA-3' and 5'-TCCTTTCCTTAGCTGACACTTGT-3'; VEGF-D 5'-GCAGCCCTAGAGAAACGTG-3' and 5'-AGGTGCTGGTGTTCATACAGAT-3'; PlGF 5'-TGCGGCGATGAGAATCTGC-3' and 5'-AGCGAACGTGCTGAGAGAAC-3'; COX2 5'-TTGCTGGAACATGGAATTACC-3' and 5'-TGCCTGCTCTGGTCAATG-3'; 5-LOX 5'-TGGCGCGGTGGATTCATAC-3' and 5'-CAGGGGAACTCGATGTAGTCC-3'; GLUT-1 5'-CTTTGTGGCCTTCTTTGAAGT-3' and 5'-CCACACAGTTGCTCCACAT-3'; CAIX 5'-GGAAGGCTCAGAGACTCA-3' and 5'- CTTAGCACTCAGCATCAC-3'. All samples were assayed in triplicate. Relative expression values were calculated using the 2(-delta delta C(T)) method and were normalized against reference genes: tata-binding protein (*TBP*) and succinate dehydrogenase complex subunit A (*SDHA*) (primers: TBP 5'-CGGCTGTTTAACTTCGCTTC-3' and 5'-CACACGCCAAGAAACAGTGA-3'; SDHA 5'-TGGGAACAAGAGGGCATCTG-3' and 5'-CCACCACTGCATCAAATTCATG-3'). In these calculations we took into account the PCR efficiency of the individual PCR reactions, calculated on the basis of linear regression as described in Ruijter et al [22]. For the comparison between healthy colon biopsies and resections, the normalized relative expression values were scaled against the median of the healthy biopsies (median of biopsies set to 1). The specificity of amplification was confirmed by evaluation of the melting curves.

| Variable | Number of patients | |
|---|---|---|
| | **Biopsy** | **Resection** |
| Sex | | |
| Male | 22 | 25 |
| Female | 16 | 14 |
| Age at diagnosis | | |
| Median age (range, years) | 70 (39–85) | 67 (39–84) |
| Site of tumor | | |
| Sigmoid | 20 | 14 |
| Colon descendens | 2 | 2 |
| Colon transversum | 1 | 4 |
| Hepatic flexure | 1 | 3 |
| Colon ascendens | 5 | 5 |
| Caecum and valve of Bauhin | 8 | 7 |
| Not specified | 1 | 4 |
| Tumor grade | | |
| Low | 3 | 5 |
| Moderate | 18 | 24 |
| High | 12 | 7 |
| Unknown | 5 | 3 |
| Dukes classification | | |
| Dukes' A | 3 | 8 |
| Dukes' B | 15 | 12 |
| Dukes' C | 6 | 12 |
| Dukes' D | 13 | 5 |
| Unknown | 1 | 2 |
| T category | | |
| T1-T2 | 3 | 8 |
| T3-T4 | 26 | 29 |
| Tx | 9 | 2 |
| Lymphatic spread | | |
| N0 | 18 | 21 |
| N+ | 10 | 16 |
| Nx | 10 | 2 |
| Metastasis | | |
| M0/Mx | 25 | 34 |
| M+ | 13 | 5 |

**Table 4.1 - Clinicopathological features of the colon carcinoma patients**

## Statistical analysis

Statistical analysis was performed using the GraphPad Prism® software (GraphPad Software Inc., La Jolla, California, USA). Statistical significance of comparisons between two independent groups was determined with the two-tailed Mann-Whitney U test. The comparison between paired samples was performed with the Wilcoxon signed-rank test. The accuracy of the markers was determined with receiver operator characteristic curves (ROC). The statistical significance of the difference between two areas under the ROC curves was calculated by the method of DeLong et al. and performed with MedCalc® software (MedCalc Software, Mariakerke, Belgium) [23]. Significant p-values were ranked as $p<0.05$ (*), $p<0.01$ (**) and $p<0.001$ (***).

### 4.1.3 Results

**Surgical resection induces hypoxic cellular stress in healthy colon tissue**

To examine to what extent the sampling procedure (biopsy *versus* surgical resection) may affect the overall condition of the sampled tissue, we analyzed the mRNA expression of COX2 and 5-LOX in samples of healthy colon tissue. As shown in figure 4.1A , expression levels of *COX2*, an inflammation and hypoxia responsive gene used here as a biomarker of cellular stress, were significantly higher in resections than in biopsies. Also the expression levels of *GLUT-1* and *CAIX*, two hypoxia markers, were significantly increased in resected samples compared to biopsy samples (Figure 4.1C-D). Finally, the expression levels of *5-LOX*, included here as a control gene induced by cellular stress but insensitive to hypoxia, were identical in the two groups of samples (Figure 4.1B). Combined, these results indicate the induction by the surgical resection procedure of hypoxic cellular stress in the resected tissue.

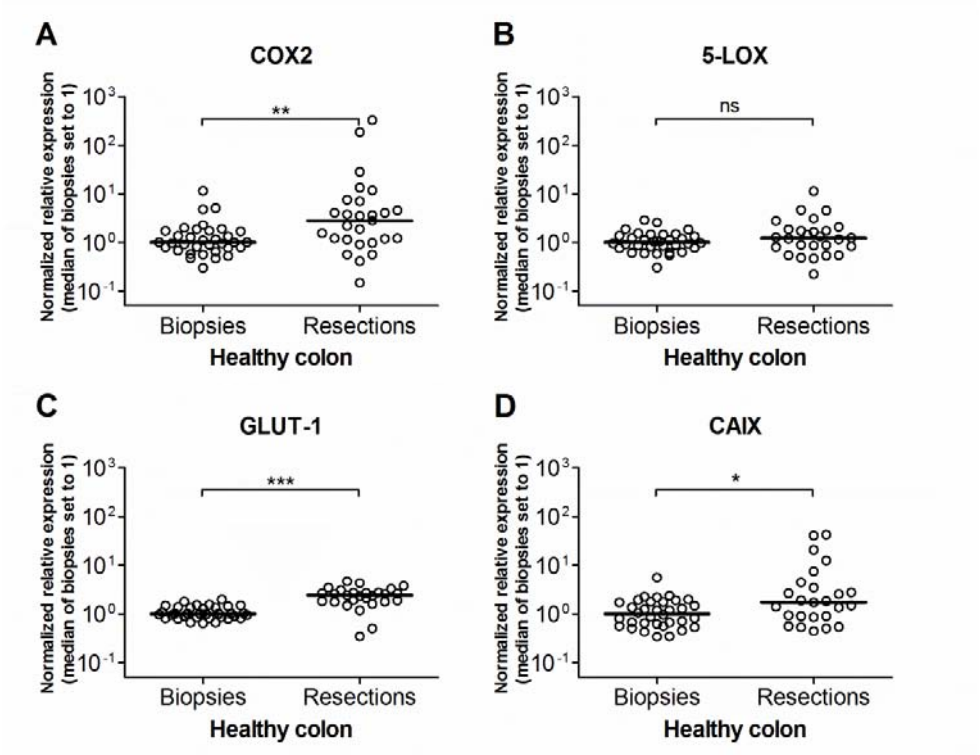


**Figure 4.1 – Effect of sampling method on the expression of inflammatory and hypoxic stress responsive genes in healthy colon tissue samples.** Relative mRNA expression levels of the inflammatory eicosanoid enzymes COX2 **(A)** and 5-LOX **(B)** and of the hypoxia markers GLUT-1 **(C)** and CAIX **(D)** are shown for healthy colon biopsy and healthy colon resection samples. Expression levels were normalized against reference genes TBP and SDHA and were scaled against the median of the biopsy samples (median set to 1). Expression data are depicted as scatter plots of the values obtained for each individual sample. The horizontal line represents the median; ns: not significant; *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$ with Mann-Whitney U Test.

### Surgical resection increases expression levels of VEGF family members in healthy colon tissue

We next determined whether the occurrence of surgery-related hypoxic stress in resected healthy tissue samples was reflected in the expression levels of the individual VEGF family members. As shown in figure 4.2, highly significant ($p<0.001$) differences between resected and biopsy healthy colon samples were observed for all the VEGF family members. For these genes, the median expression levels were two- to three-fold (VEGF-A, -B, -C and PlGF) higher in resected than in biopsy samples, up to a striking 22-fold increase for VEGF-D.

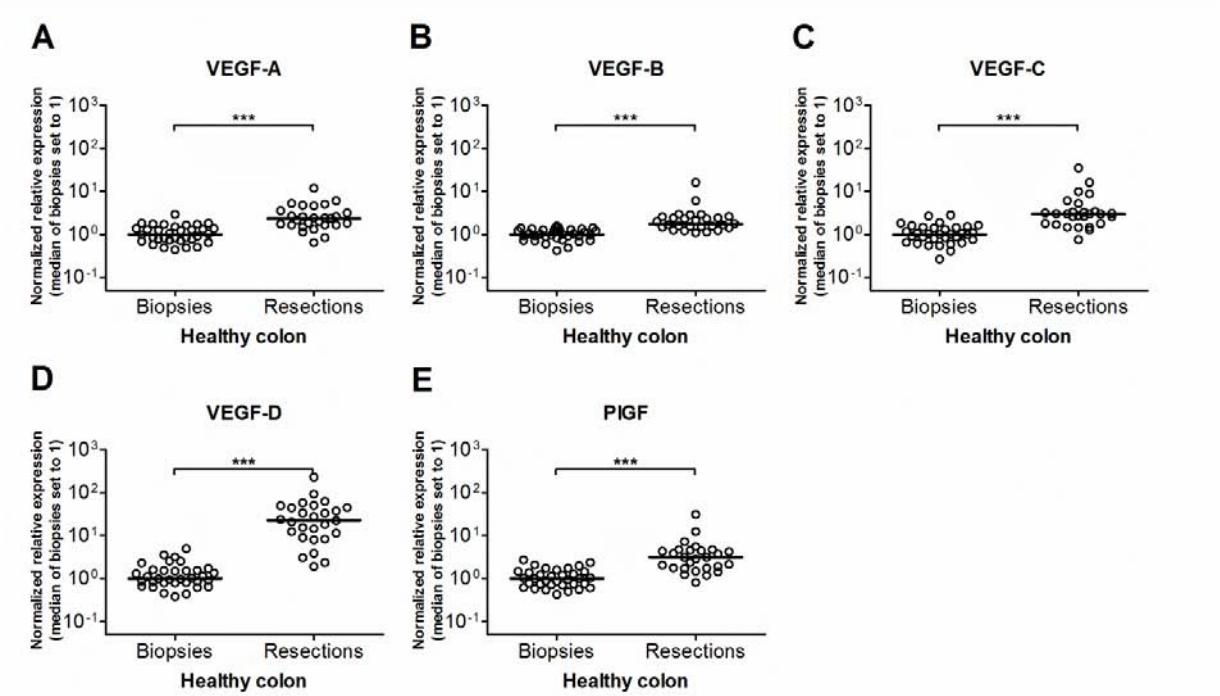


**Figure 4.2 – Effect of sampling method on the expression of VEGF family members in healthy colon tissue samples.** Relative mRNA expression levels of VEGF-A **(A)**, VEGF-B **(B)**, VEGF-C **(C)**, VEGF-D **(D)** and PlGF **(E)** are shown for healthy colon biopsy and healthy colon resection samples. Expression levels were normalized against reference genes TBP and SDHA and were scaled against the median of the healthy colon samples (median set to 1). Expression data are depicted as scatter plots of the values obtained for each individual sample. The horizontal line represents the median; ***: $p<0.001$ with Mann-Whitney U Test.

### The sampling procedure affects the biomarker read-out of VEGF family members

We next assessed the extent to which the sampling-induced differences in VEGF gene expression observed in healthy tissue affected the magnitude of the difference between healthy and carcinoma tissue. To that end, we compared VEGF gene induction in colon carcinoma to matched healthy tissue samples obtained by biopsy or by surgical resection. Expression levels of

*VEGF-A* were significantly induced in carcinoma tissues towards healthy tissues independent of the sampling method (Figure 4.3A). However, for the other VEGF family members, the magnitude of the difference between healthy and carcinoma tissue in resection samples was affected by the increment of expression in healthy tissue caused by the surgical sampling procedure. For *VEGF-B*, *VEGF-C* and *PlGF*, this resulted in reduced expression differences between healthy and carcinoma tissue in resected samples (Figure 4.3B, C and E). The consequences are most pronounced for VEGF-B that albeit significantly induced in biopsy carcinoma samples, no longer showed significance in carcinoma samples obtained by surgical resection (Figure 4.3B). A similar sampling procedure induced turnaround of biomarker value is observed for VEGF-D, although in an opposite direction. Here, the pronounced increase in the expression of *VEGF-D* in healthy resected tissue as opposed to the near absence of such an increase in carcinoma tissue resulted in a highly significant underexpression of *VEGF-D* in carcinoma resection samples (Figure 4.3D). On the contrary, in biopsy samples no difference in *VEGF-D* expression between healthy colon and colon carcinoma samples was observed.

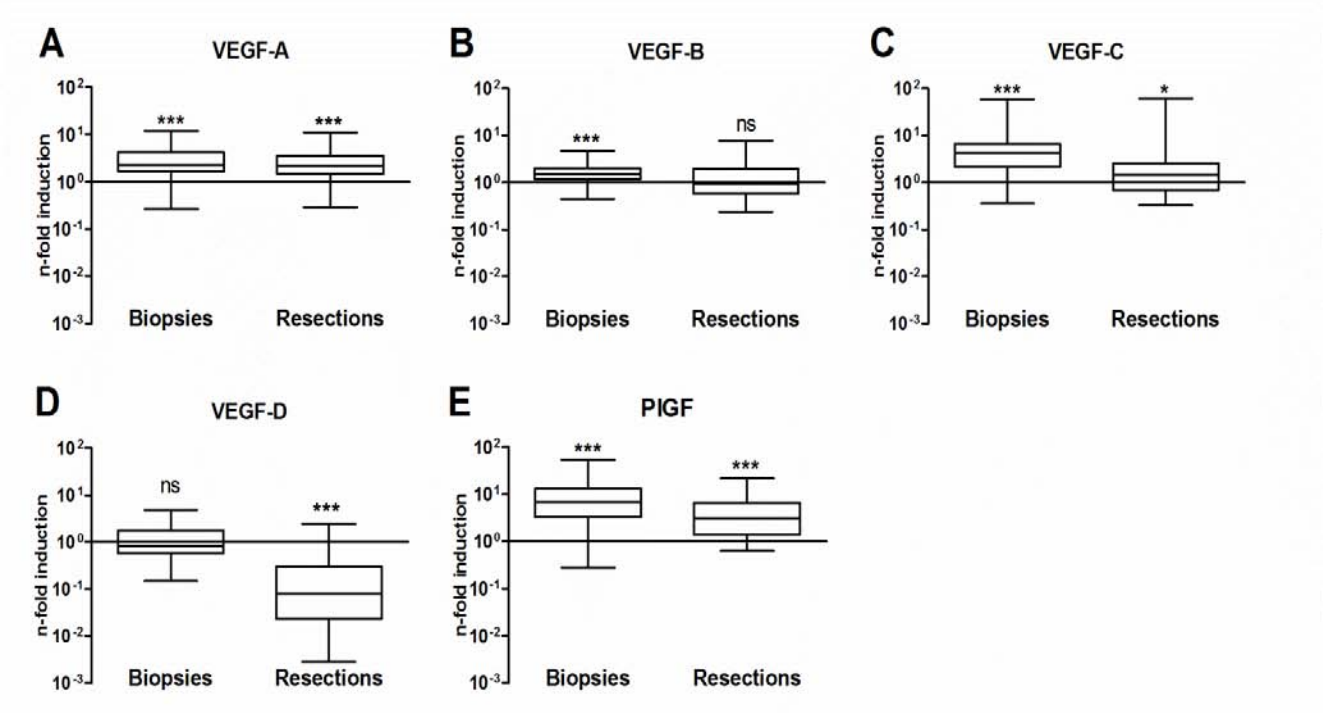


**Figure 4.3 – Influence of sampling method on the biomarker read-out of VEGF family members.** n-Fold induction levels in carcinoma samples of VEGF-A **(A)**, VEGF-B **(B)**, VEGF-C **(C)**, VEGF-D **(D)** and PlGF **(E)** are shown. The n-fold induction value represents the ratio of the expression value of the carcinoma sample against the expression value of the paired healthy sample. The box represents the median with interquartile range and the whiskers represent minimum and maximum ratios. ns: not significant; *:$p<0.05$; ***: $p<0.001$ with Wilcoxon signed-rank test.

**Cancer biomarker accuracy of VEGF family members**

Receiver-operating characteristics (ROC) analysis is commonly used to assess the reliability and accuracy of potential biomarkers. ROC-based assessment of the individual VEGF family members as biomarkers for colon cancer identified overexpression of PlGF (AUC 0.9342) as the most effective mRNA-marker for samples obtained by biopsy with VEGF-A (AUC 0.8760) and VEGF-C (AUC 0.8977) following as close seconds (figure 4.4). This ranking however changes dramatically when considering samples obtained by resection. Here, underexpression of VEGF-D emerges as the most potent biomarker with an AUC of 0.9047 ($p<0.0001$) and a ROC-curve significantly different ($p<0.0001$) from the biopsy curve (Figure 4.4D). Overexpression of VEGF-A (AUC 0.8573) now precedes PlGF (AUC 0.8231), VEGF-C (AUC 0.6200) and especially VEGF-B (AUC 0.5621) shows strongly reduced accuracy as colon cancer mRNA-marker (Figure 4.4).

### 4.1.4 Discussion

Biomarker expression profiles have become a valuable tool in diagnostic research, patient management and cancer therapy. We explored the influence of different sampling methods on the expression of VEGF family biomarkers in colon cancer. Samples obtained by either biopsy or surgical resection were compared for the differential expression of *VEGF-A*, *VEGF-B*, *VEGF-C*, *VEGF-D* and *PlGF*. To examine the occurrence of cellular stress caused by the sampling procedure, the expression levels of the eicosanoid enzymes *COX2* and *5-LOX* were quantified in healthy colon tissue. COX2 is a key inflammatory enzyme, and its expression is strongly induced by NF-κB and HIF-1 transcription factors in response to inflammatory insults and hypoxic growth conditions, respectively [19, 24-28]. In contrast, expression of 5-LOX is largely insensitive to hypoxia but is similarly induced by various inflammatory insults [19, 29, 30]. Strikingly, we observed a pronounced expression increment of *COX2* in healthy colon resection samples relative to healthy biopsy samples. This was not the case for *5-LOX*. This differential expression pattern of *COX2* as opposed to *5-LOX* indicates that considerably more hypoxic stress may be present in resection samples than in samples obtained by biopsy. The presence of hypoxia in resection samples was further substantiated by the significantly increased expression in resection samples of the hypoxia markers GLUT-1 and CAIX. There is a large difference in the time needed to obtain samples by the two procedures. Whereas the collection of colon biopsies

requires only minutes, surgical resection takes 30 to 90 minutes, during which the colon is clamped off. This cuts off blood circulation and oxygen delivery and could cause hypoxia in the clamped colon. The observed increment in COX2, GLUT-1 and CAIX mRNA levels in healthy colon tissue resections might therefore be a direct consequence of the clamping of part of the colon inducing a hypoxic stress signal.

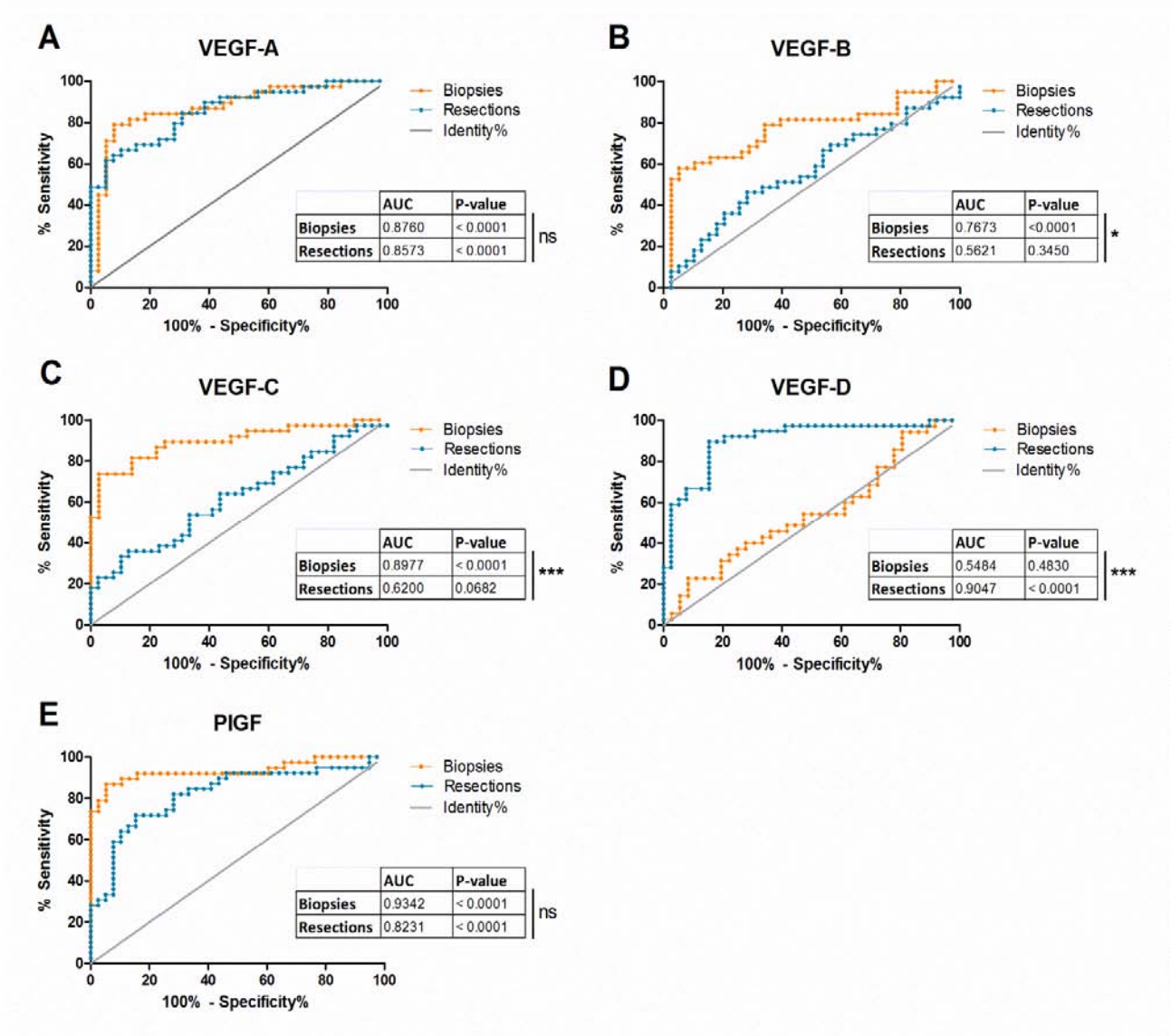


**Figure 4.4 – ROC-analysis of the biomarker accuracy of VEGF family members for biopsy and resection samples.** ROC-curves of VEGF-A **(A)**, VEGF-B **(B)**, VEGF-C **(C)**, VEGF-D **(D)** and PlGF **(E)** are shown for biopsy and resection samples. The ROC-curves represent the sensitivity and specificity of the individual VEGF family members as colon carcinoma biomarkers. The insert gives the area under the curve (AUC), which quantifies the ability of the marker to distinguish between healthy colon and colon carcinoma. The accompanying p-value tests the null hypothesis, namely, that the AUC equals 0.50 and thus the biomarker is incompetent. The identity-line (Identity%) represents the null hypothesis. *: $p<0.05$; ***: $p<0.001$ calculated with the method of DeLong et al. [23].

VEGF-A is readily induced by COX2 derived prostaglandins such as $PGE_2$ [31, 32]. Concomitant with the clear induction of COX2 mRNA in resected healthy tissue, *VEGF-A* expression levels were increased in healthy tissue resections. However, besides VEGF-A also other VEGF family members showed significant mRNA expression increments in resected healthy tissue ranging from 2-3 fold (VEGF-B, VEGF-C, PlGF) up to 22-fold (VEGF-D). Two recent reports described the induction during hypoxia of these VEGF family members in lung and lymphatic endothelial cells [33, 34]. It is therefore likely that the combined action of clamping-induced hypoxia and COX2 derived prostaglandins are at the basis of the increased mRNA expression of VEGF-A as well as of the other VEGF family members we observed in resected healthy colon samples.

A determining factor in defining a biomarker is its accuracy in differentiating a healthy from a diseased state. Therefore, we assessed the ability of the individual VEGF members to discriminate between healthy and cancerous colon tissue and the influence of the sampling method on this ability. Although the cohort size (n=77) so far is rather limited, VEGF-A and PlGF emerged as potential mRNA-markers discriminating with relatively high accuracy between healthy and carcinoma tissue in samples obtained by biopsy or by surgical resection. Our results confirm previous studies reporting significantly increased expression levels of *VEGF-A* in colon carcinoma samples compared to healthy tissue [5-9]. The same conclusion holds true for PlGF. Of all VEGF family members, PlGF emerged from our ROC-analysis as the most accurate biomarker in both the sampling methods and was even more accurate than VEGF-A in biopsies. It is therefore remarkable that PlGF has received less attention than other VEGF members in colon carcinoma. Wei and colleagues studied resection samples from colorectal carcinoma patients and also documented increased PlGF mRNA expression levels and their association with reduced survival [9]. A similar result was obtained for both PlGF isoforms, PlGF-1 and PlGF-2, by Escudero-Esparza and colleagues [35]. Our observations further confirm these findings.

For VEGF-B, VEGF-C and VEGF-D we observed a significant impact of the sampling procedure on the mRNA expression levels in healthy versus colon carcinoma tissues. Table 4.2 compares our observations with previously reported data taking into account the reported sampling method but also other potential confounding factors such as the inclusion or not of rectal samples.

| VEGF family member | Own data | | Published data | | | |
|---|---|---|---|---|---|---|
| | Δ expression | | Δ expression | Sampling method | Other confounders | Refs |
| | Resection | Biopsy | | | | |
| **VEGF-B** | = | ↗ *** | = | Resection | - | [5] |
| | | | = | Resection | Rectal incl. | [7] |
| | | | = | Resection | Rectal incl. | [8] |
| **VEGF-C** | ↗ * | ↗ *** | = | Resection | - | [5] |
| | | | ↗ | n.s. | Rectal incl. | [6] |
| | | | ↗ | Resection | Rectal incl. | [7] |
| | | | = | Resection | Rectal incl. | [8] |
| | | | = | Biopsy | Rectal incl. | [14] |
| **VEGF-D** | ↘ *** | = | ↘ | n.s. | Rectal incl. | [6] |
| | | | ↘ | Resection | Rectal incl. | [7] |
| | | | ↘ | Resection | Rectal incl. | [8] |
| | | | ↘ | Biopsy | Rectal incl. | [14] |

Δ expression: differential expression in carcinoma samples compared to healthy tissue samples
↗: significantly increased
↘: significantly decreased
=: no significance
n.s.: not specified
Rectal incl.: rectal samples included in the analysis
*: $p<0.05$ with Wilcoxon signed rank test
***: $p<0.001$ with Wilcoxon signed rank test

**Table 4.2 - Comparison of expression data for VEGF-B, VEGF-C and VEGF-D with previously published reports.**

Previous studies did not reveal overexpression of *VEGF-B* in colon carcinoma (Table 4.2). Also we did not observe increased VEGF-B mRNA levels in samples obtained by surgical resection. However, this lack of overexpression appears to be a consequence of the surgical sampling method rather than a characteristic intrinsic to colon carcinoma. This conclusion is based on the pronounced expression increment we observed in carcinoma tissue obtained by colonoscopic biopsy. These opposite results clearly identify the strong impact of the sampling procedure on VEGF-B mRNA-levels and challenge the conclusions of previous studies using samples obtained by surgical resection [5, 7, 8]. VEGF-C resembles VEGF-B in the impact of the clinical sampling method, showing a weak overexpression in resections as opposed to a pronounced, highly significant overexpression in biopsies (Table 4.2). Two out of five previously published reports

similarly documented increased *VEGF-C* expression levels. Other reports using either biopsy or resected material failed however to detect significant changes. These conflicting data may be due to confounding factors other than the sampling method, namely the inclusion of rectal samples in these studies. Because radiotherapy prior to surgery is standard procedure in rectal cancer, we excluded such patients from our study. Finally, also VEGF-D shows a strong impact of the sampling method on its differential mRNA expression (Table 4.2). Here however, resected tissue samples show a pronounced underexpression as opposed to the absence of a differential expression in biopsy samples. This surgery-created signature again emphasizes the importance of taking into account the clinical procedure used for colon tissue sampling when performing colon cancer expression studies.

Our study included a total of 77 patients. Though this is a large cohort, clearly it is not large enough to exclude biases due to type I error. To detect type I errors, we statistically analyzed the likelihood that group-related disparities in gender, tumor grade, sample location and age confounded the conclusions of our study. As shown in the supplementary data (tables S4.1, S4.2, S4.3, S4.4 and S4.5), we did not detect specific biases that could contribute to the observed differential gene expression patterns. Yet, expansion of this study to a larger patient cohort may help to further corroborate our findings of direct relevance for colon cancer diagnosis and basic research.

**Conclusions**

Our comparative gene mRNA expression analysis of healthy and carcinoma colon tissue shows that the sampling procedure - surgical resection *versus* colonoscopic biopsy - has an important impact on the read-out of VEGF family members as potential colon cancer mRNA-markers. The sampling-induced modulation of *VEGF* gene expression profiles could be related to cellular stress caused by hypoxia elicited in resected tissue samples by clamping of blood vessels during surgery. The higher sensitivity of healthy tissue to surgery-induced cellular stress compared to the relative insensitivity of carcinoma tissue affected to different degrees the reliability of individual VEGF-members as mRNA-markers for colon carcinoma. Therefore, samples obtained by biopsy provide a more reliable VEGF mRNA-marker read-out than samples obtained by surgical resection.

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## SUPPLEMENTAL DATA

| Gene | Biopsies | | | | Resections | | | |
|---|---|---|---|---|---|---|---|---|
| | Healthy colon | | Colon carcinoma | | Healthy colon | | Colon carcinoma | |
| | p-Value | Sign diff? [1] | p-Value | Sign diff? [1] | p-Value | Sign diff? [1] | p-Value | Sign diff? [1] |
| **COX2** | 0.6119 | no | - | - | 0.4253 | no | - | - |
| **5-LOX** | 0.9860 | no | - | - | 0.3413 | no | - | - |
| **GLUT-1** | 0.9025 | no | - | - | 0.2268 | no | - | - |
| **CAIX** | 0.6618 | no | - | - | 0.3159 | no | - | - |
| **VEGF-A** | 0.1008 | no | 0.8708 | no | 0.9184 | no | 0.9417 | no |
| **VEGF-B** | 0.0948 | no | 0.5643 | no | 0.6290 | no | 0.4732 | no |
| **VEGF-C** | 0.1315 | no | 0.3831 | no | 0.0868 | no | 0.8721 | no |
| **VEGF-D** | 0.0426* | * | 0.0765 | no | 0.7363 | no | 0.4642 | no |
| **PlGF** | 0.2312 | no | 0.9176 | no | 0.8952 | no | 0.9184 | no |

[1] Sign diff?: Significant difference between samples from male or female patients?
Biopsies: male: n=22; female: n=16
Resections: male: n=25; female: n=14

**Table S4.1 - Comparison of expression levels in male versus female patients** with Mann-Whitney test. *: p<0.05

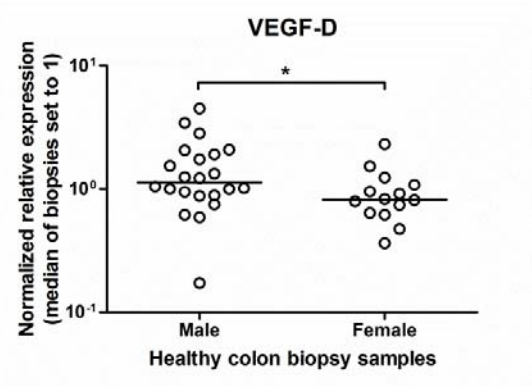


**Figure S4.1 - Comparison of VEGF-D expression levels in male versus female patients** in healthy colon biopsy samples with Mann-Whitney test. *: p<0.05

| Gene | Biopsies | | | | Resections | | | |
|---|---|---|---|---|---|---|---|---|
| | Healthy colon | | Colon carcinoma | | Healthy colon | | Colon carcinoma | |
| | p-Value | Sign diff? [1] | p-Value | Sign diff? [1] | p-Value | Sign diff? [1] | p-Value | Sign diff? [1] |
| **COX2** | 0.0883 | no | - | - | 0.0397 | * | - | - |
| **5-LOX** | 0.4667 | no | - | - | 0.1646 | no | - | - |
| **GLUT-1** | 0.5942 | no | - | - | 0.7709 | no | - | - |
| **CAIX** | 0.3980 | no | - | - | 0.6819 | no | - | - |
| **VEGF-A** | 0.5775 | no | 0.3662 | no | 0.6067 | no | 0.2818 | no |
| **VEGF-B** | 0.8955 | no | 0.8589 | no | 0.6085 | no | 0.6771 | no |
| **VEGF-C** | 0.9849 | no | 0.5805 | no | 0.1073 | no | 0.0321 | * |
| **VEGF-D** | 0.4946 | no | 0.6100 | no | 0.6195 | no | 0.1850 | no |
| **PlGF** | 0.0394 | * | 0.9056 | no | 0.2856 | no | 0.8994 | no |

[1] Sign diff?: Significant difference between samples from different tumor grade?
Biopsies: low grade: n=3; moderate grade: n=18; high grade: n=12
Resections: low grade: n=5; moderate grade: n=24; high grade: n=7

**Table S4.2 - Comparison of expression levels in colon carcinoma with tumor grade low versus moderate versus high** with Kruskal Wallis test. *: $p<0.05$

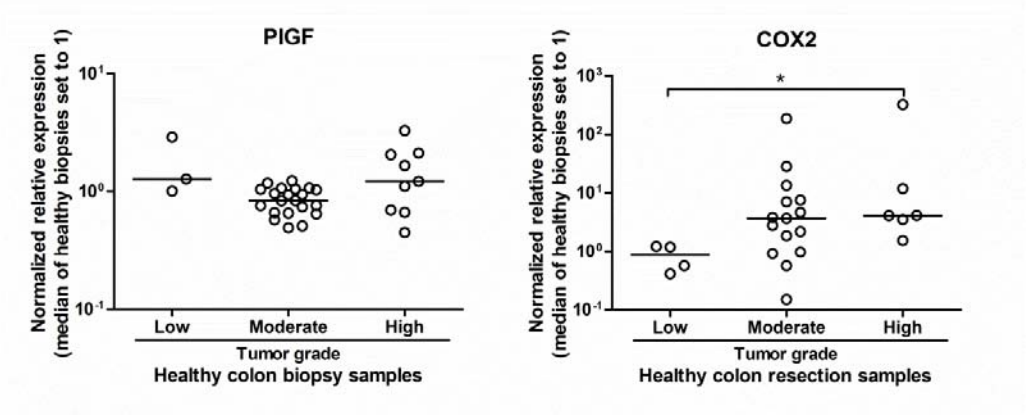


**Figure S4.2 - Comparison of expression levels in colon carcinoma with tumor grade low versus moderate versus high** for PlGF in healthy colon biopsy samples and for COX2 in healthy colon resection samples with Kruskal Wallis test. *: $p<0.05$. For PlGF, the difference between the medians is statistically significant ($p<0.05$), but Dunn's multiple comparison test detects no specific significant difference between two groups.

| Gene | Biopsies | | | | Resections | | | |
|---|---|---|---|---|---|---|---|---|
| | Healthy colon | | Colon carcinoma | | Healthy colon | | Colon carcinoma | |
| | p-Value | Sign diff?[1] | p-Value | Sign diff? [1] | p-Value | Sign diff? [1] | p-Value | Sign diff? [1] |
| **COX2** | 0.6734 | no | - | - | 0.6011 | no | - | - |
| **5-LOX** | 0.3685 | no | - | - | 0.0613 | no | - | - |
| **GLUT-1** | 0.3680 | no | - | - | 0.1770 | no | - | - |
| **CAIX** | 0.6838 | no | - | - | 0.6578 | no | - | - |
| **VEGF-A** | 0.6942 | no | 0.7199 | no | 0.0800 | no | 0.3722 | no |
| **VEGF-B** | 0.7275 | no | 0.6361 | no | 0.0337 | * | 0.1204 | no |
| **VEGF-C** | 0.8051 | no | 0.5012 | no | 0.1017 | no | 0.1844 | no |
| **VEGF-D** | 0.4686 | no | 0.7552 | no | 0.1827 | no | 0.6879 | no |
| **PlGF** | 0.7782 | no | 0.7614 | no | 0.0993 | no | 0.1650 | no |

[1] Sign diff?: Significant difference between samples with different Dukes classification?
Biopsies: A: n=3; B: n=15; C: n=6; D: n=13
Resections: A: n=8; B: n=12; C: n=12; D: n=5

**Table S4.3 - Comparison of expression levels in colon carcinoma with Dukes classification A versus B versus C versus D** with Kruskal Wallis test. *: $p<0.05$

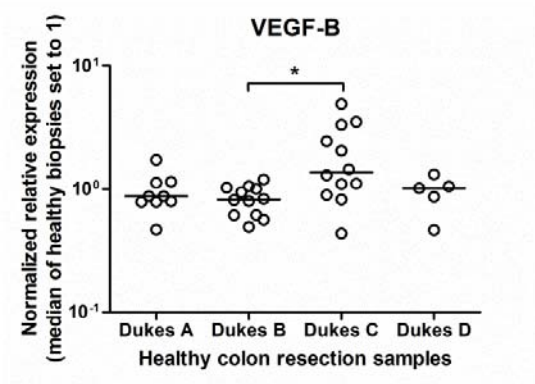


**Figure S4.3 - Comparison of VEGF-B expression levels in colon carcinoma with Dukes classification A versus B versus C versus D** in healthy colon resection samples with Kruskal Wallis test. *: $p<0.05$

| Gene | Biopsies | | | | Resections | | | |
|---|---|---|---|---|---|---|---|---|
| | Healthy colon | | Colon carcinoma | | Healthy colon | | Colon carcinoma | |
| | p-Value | Sign diff? [1] | p-Value | Sign diff? [1] | p-Value | Sign diff? [1] | p-Value | Sign diff? [1] |
| **COX2** | 0.1729 | no | - | - | 0.7113 | no | - | - |
| **5-LOX** | 0.3979 | no | - | - | 0.9803 | no | - | - |
| **GLUT-1** | 0.5928 | no | - | - | 0.0887 | no | - | - |
| **CAIX** | 0.0136 | * | - | - | 0.7861 | no | - | - |
| **VEGF-A** | 0.7927 | no | 0.6827 | no | 0.1227 | no | 0.9210 | no |
| **VEGF-B** | 0.4835 | no | 0.9767 | no | 0.0490 | * | 0.3013 | no |
| **VEGF-C** | 0.2674 | no | 0.6197 | no | 0.0490 | * | 0.2514 | no |
| **VEGF-D** | 0.2540 | no | 0.3818 | no | 0.1127 | no | 0.1975 | no |
| **PlGF** | 0.7703 | no | 0.6404 | no | 0.6403 | no | 0.1879 | no |

[1] Sign diff?: Significant difference between samples from patients of ≤70 years or >70 years?
Biopsies: ≤70 years: n=19; >70 years: n=19
Resections: ≤70 years: n=22; >70 years: n=17

**Table S4.4 - Comparison of expression levels in patients younger than 70 years or of 70 years versus patients older than 70** with Mann-Whitney test. *: p<0.05

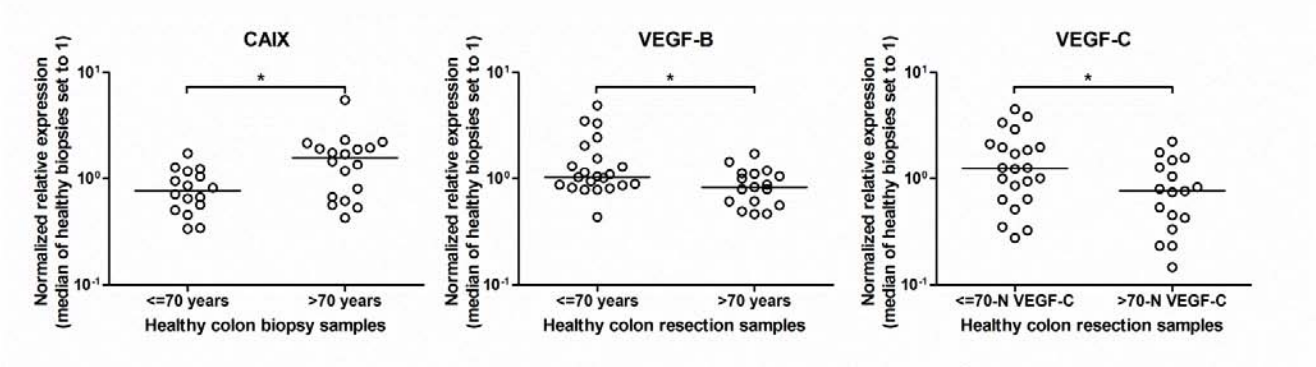


**Figure S4.4 - Comparison of expression levels in patients younger than 70 years or of 70 years versus patients older than 70** for CAIX in healthy colon biopsy samples and for VEGF-B and VEGF-C in healthy colon resections with Mann-Whitney test. *: p<0.05

| Gene | Biopsies | | | | Resections | | | |
|---|---|---|---|---|---|---|---|---|
| | Healthy colon | | Colon carcinoma | | Healthy colon | | Colon carcinoma | |
| | p-Value | Sign diff?[1] | p-Value | Sign diff?[1] | p-Value | Sign diff?[1] | p-Value | Sign diff?[1] |
| **COX2** | n/a | n/a | - | - | n/a | n/a | - | - |
| **5-LOX** | n/a | n/a | - | - | n/a | n/a | - | - |
| **GLUT-1** | n/a | n/a | - | - | n/a | n/a | - | - |
| **CAIX** | n/a | n/a | - | - | n/a | n/a | - | - |
| **VEGF-A** | n/a | n/a | 0.0671 | no | n/a | n/a | 0.7597 | no |
| **VEGF-B** | n/a | n/a | 0.5926 | no | n/a | n/a | 0.7546 | no |
| **VEGF-C** | n/a | n/a | 0.2775 | no | n/a | n/a | 0.7456 | no |
| **VEGF-D** | n/a | n/a | 0.8341 | no | n/a | n/a | 0.7937 | no |
| **PlGF** | n/a | n/a | 0.5477 | no | n/a | n/a | 0.9067 | no |

[1] Sign diff?: Significant difference between samples from different tumor sites?
Biopsies: (A): n=8; (B): n=9; (C): n=20
Resections: (A): n=7; (B): n=14; (C): n=14

**Table S4.5 - Comparison of expression levels in healthy colon and colon carcinoma samples from different tumor sites** ((A) caecum and Valve of Bauhin versus (B) colon ascendens, transversum, descendens and hepatic flexure versus (C) sigmoid) with Kruskal Wallis test. n/a: not applicable.

## 4.2 Additional data

### 4.2.1 Similar inflammatory and angiogenic expression profiles for proximal and distal healthy colon samples

To verify if the distance away from the tumor influences the expression profiles from healthy colon tissue samples in a similar way as the clinical procedure, paired proximal and distal healthy colon tissue samples were analyzed by RT-qPCR in a pilot study. The proximal sample biopsies were taken at approximately 5 cm from the tumor, whereas the distal biopsies were sampled as far away from the tumor as feasible, at least 20-40 cm away. The experimental setup was identical as described in the material and methods section of section 4.1.

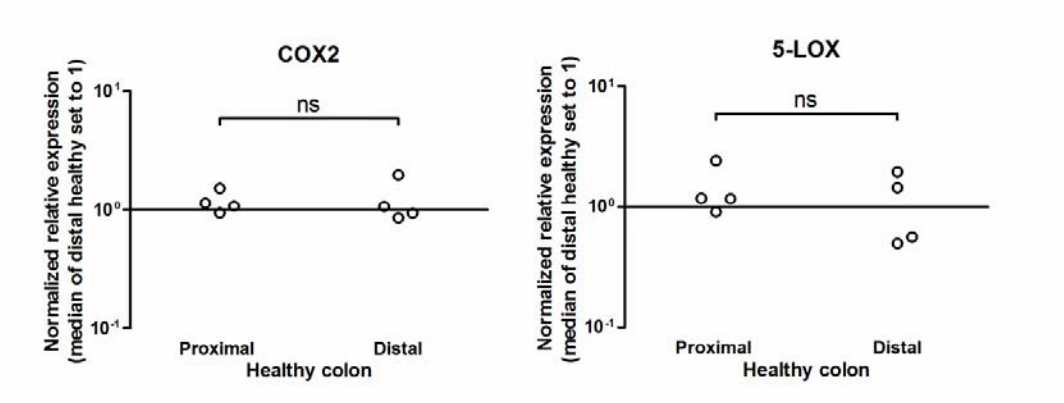


**Figure 4.5 - Comparison of COX2 and 5-LOX expression levels between proximal and distal healthy colon tissue samples.** Relative mRNA expression levels of the inflammatory eicosanoid enzymes COX2 and 5-LOX are shown for proximal and distal healthy colon tissue samples. Expression levels were normalized against reference genes TBP and SDHA and were scaled against the median of the distal samples (median set to 1). Expression data are depicted as scatter plots of the values obtained for each individual sample. ns: not significant with Wilcoxon signed-rank test.

The expression levels of the eicosanoid enzymes *COX2* and *5-LOX*, analyzed as markers for inflammation and cellular stress, showed no difference between proximal and distal healthy colon tissue samples (Figure 4.5). Likewise, VEGF family members were expressed at similar levels in proximal and distal healthy colon tissue samples (Figure 4.6). Although the sample size was small (n=4), these results give no indication that expansion to a larger patient cohort may give any significant differences. Therefore, we believe that there is no difference in expression of the studied inflammatory and angiogenic genes between proximal – representing the "routine" healthy control samples – and distal healthy colon tissue samples.

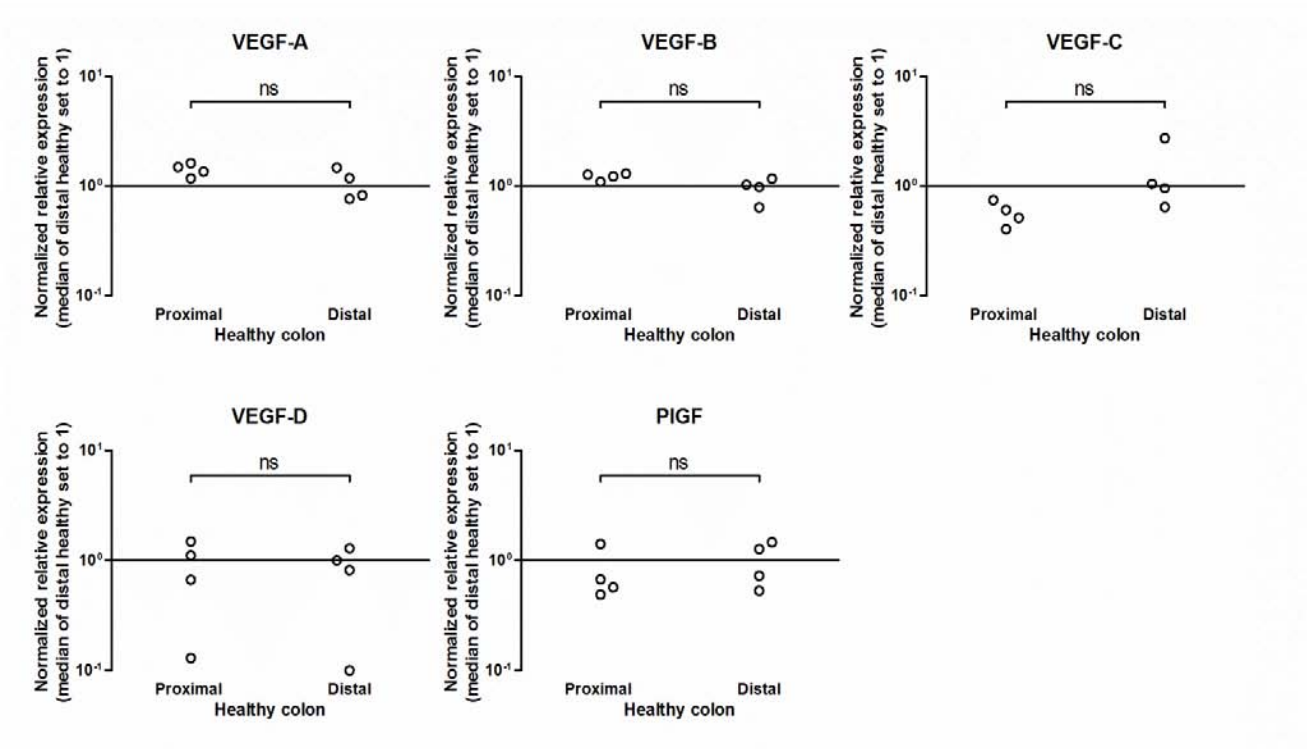


**Figure 4.6 - Comparison of VEGFs expression levels between proximal and distal healthy colon tissue samples.** Relative mRNA expression levels of VEGF-A, VEGF-B, VEGF-C, VEGF-D and PlGF are shown for proximal and distal healthy colon tissue samples. Expression levels were normalized against reference genes TBP and SDHA and were scaled against the median of the distal samples (median set to 1). Expression data are depicted as scatter plots of the values obtained for each individual sample. ns: not significant with Wilcoxon signed-rank test.

# Chapter 5

# Evolution of the VEGF family during the progression of colon cancer

**Contributions**

This study was conceived and designed by Sarah Pringels, Nancy Van Damme, Johan Grooten and Marc Peeters.

All technical experiments were performed by Sarah Pringels.

The acquisition of patient data and clinical samples was coordinated by Nancy Van Damme, Karen Geboes and Evi Mampaey.

Expert advice and support on RT-qPCR expression analysis were provided by Bram De Craene.

## 5.1 Stage-specific cumulative expression of VEGF family members during colon cancer progression

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### Abstract

The vascular endothelial growth factor family members (VEGF-A, -B, -C, -D and placental growth factor (PlGF)) are key players in the development, maintenance and remodeling of the vascular and lymphatic microcirculation as well as in the outgrowth of solid tumors. However, data on the expression of VEGF family members in colon carcinoma and during its malignant progression are scattered and often contradictory, rendering it almost impossible to obtain a global picture. Therefore, we now performed a comprehensive analysis of the mRNA expression signatures of all VEGF family members at the different stages of progression towards malignancy. This resulted in the identification of *VEGF-B* and *VEGF*-C as carcinoma stage-specific angiogenic genes who are upregulated only in carcinoma and metastasis samples whereas *PlGF* and *VEGF-A* are overexpressed compared to matched healthy colon tissue already at the stage of adenoma. An expression increment of *VEGFs* in liver metastases was observed independent of the healthy tissue calibrator, either healthy liver or healthy colon. Remarkably, the angiogenic signature of colon carcinomas was highly conserved in liver metastases but differed significantly from colon adenomas. This illustrates the occurrence of a stage-specific deviation in the expression of VEGF family members during colon carcinoma progression, *VEGF-A* and/or *PlGF* showing overexpression already at the adenoma stage and at the malignant carcinoma and metastasis stages becoming further supplemented with overexpressed *VEGF-B* and *VEGF-C.*

### 5.1.1 Introduction

Angiogenesis and lymphangiogenesis are crucial processes implicated in normal physiology but also in pathological conditions such as inflammation and cancer. In cancer, the formation of new blood vessels to deliver nutrients and oxygen to the growing tumor constitutes a vital process in solid tumor development and progression [1, 2]. Therefore, angiogenesis has become a main target for anti-tumoral therapy. Blocking the development of tumor vasculature (angiostatic therapy) and/or disrupting established tumor vasculature not only limits the outgrowth of solid tumors but may also prevent the formation of distant metastases [1-4]

A central constituent in the development, maintenance and remodeling of the vascular microcirculation is vascular endothelial growth factor (VEGF). Since its identification in 1983 [5], the family of vascular active cytokines has grown to include VEGF-A, VEGF-B, VEGF-C, VEGF-D and placental growth factor (PlGF) [6]. VEGF-A, the member first discovered, induces endothelial cell proliferation, survival and migration as well as tube formation [7]. VEGF-A expression is readily induced during inflammation but also in most solid tumors, including colon cancer [6, 8]. Neutralization of tumor-expressed VEGF-A with humanized monoclonal antibodies such as bevacizumab has resulted in significant clinical improvement of patients with colorectal, non-small cell lung, renal and breast cancer when combined with conventional fluorouracil-based chemotherapy [9]. However, the effect of VEGF-A neutralizing treatments remains modest in other cancers. In addition, resistance tends to develop after a transitory period of clinical benefit, resulting in regrowth of the tumors and progression of the disease. Angiogenic escape involving the compensatory (over)expression of VEGF-related angiogenic factors has been proposed to contribute to this limited response to VEGF-A directed angiogenic therapy [9, 10]. In line herewith, Fan et al. showed increased expression levels of VEGF-A, -B, -C and PlGF in colorectal cell lines after chronic exposure to bevacizumab [11]. In addition, bevacizumab induced reactivity to VEGF-C and VEGF-D in human brain and tumor derived endothelial cells [12]. In patients with refractory metastatic renal cell carcinoma, VEGF-A targeted angiostatic treatment concurred with increased PlGF plasma levels [13]. Next to a role for PlGF in angiogenic rescue, the VEGF-homolog may also contribute to neo-angiogenesis during solid tumor growth as indicated by the correlation of PlGF expression and preoperative serum levels with disease progression, reduced patient survival and recurrence in colorectal cancer [9, 14-16].

Increased mRNA and protein expression levels also of VEGF-B, VEGF-C and VEGF-D have been demonstrated in various cancers, including colon cancer [17-20], and have been correlated with lymphatic invasion, lymph node metastases and prognosis of patients with colon cancer [19, 21-23]. In addition, VEGF-C plasma levels have been evaluated as a predictive marker for lymphatic and venous invasion and poor outcome of colorectal cancer patients [24, 25].

Although nearly each VEGF family member has been described to be upregulated in colon cancer and/or has been correlated to clinicopathological features as described above, other studies claim unaltered expression levels in colon carcinoma of individual VEGF family members [18, 22, 23, 26-28]. This controversy is even more apparent when considering other stages of colon carcinoma, i.e. colon adenoma preceding the carcinoma stage and metastases derived from the primary colon carcinoma. In colon adenoma, VEGF-A, VEGF-B and VEGF-C expression has been reported to be unaltered compared to healthy colon or in contrary to show elevated levels [26, 27, 29]. In liver metastases, mainly VEGF-A expression has been verified. Here, VEGF-A mRNA expression levels have been reported to be either similar or higher compared to primary colon carcinoma [30, 31]. The analysis of these VEGF angiogenic factors individually and/or in small cohorts and by means of different analytical methods, likely is at the basis of these contradictory reports. Further adding to the confusion, it remains largely unclear to what extent individual carcinomas concomitantly express multiple VEGFs, thus acquiring redundancy in angiogenic function, and to what extent such a deviant trait evolves during progression from adenoma to carcinoma and metastasis. In order to circumvent these limitations, we here performed a comprehensive study determining in a single experimental setup the mRNA expression signatures of all VEGF family members during malignant progression from adenoma to carcinoma and liver metastasis.

### 5.1.2 Materials and methods

#### Biological samples

Samples were obtained from colon adenoma (n=16) and primary colon carcinoma (n= 37 ) and liver metastasis (n=23) at the Ghent University Hospital. Adenoma and carcinoma were sampled by biopsy in the infiltrating area of the growth. Histopathological examination confirmed the adenoma/carcinoma state of the tissue. From each patient, a corresponding healthy colon sample was taken from the same colon segment. None of the patients had received chemo- or

radiotherapy before colonoscopy. Liver metastasis samples from colon carcinoma patients and healthy liver tissue from the same patients were obtained by surgical resection. Seventy-eight percent of the patients had received chemotherapy before metastasectomy. Tissue samples were collected immediately after isolation in RNA*later*® Solution (Ambion/Applied Biosystems, Foster City, CA). All samples were kept at -80°C until RNA extraction. The clinicopathological features of the patients are summarized in tables 5.1, 5.2 and 5.3. All tissues were obtained following informed consent of the patients and approval of the study by the Ethics Committee of the Ghent University Hospital.

| **Variable** | **Number of patients** |
|---|---|
| Sex | |
| Male | 21 |
| Female | 16 |
| Age at diagnosis | |
| Median age (range, years) | 71 (39–85) |
| Site of tumor | |
| Sigmoid | 20 |
| Colon descendens | 1 |
| Colon transversum | 2 |
| Hepatic flexure | 1 |
| Colon ascendens | 5 |
| Caecum | 7 |
| Not specified | 1 |
| Differentiation grade | |
| Low | 12 |
| Moderate | 17 |
| High | 3 |
| Unknown | 5 |
| Dukes classification | |
| Dukes' A | 3 |
| Dukes' B | 15 |
| Dukes' C | 7 |
| Dukes' D | 12 |
| Unknown | 0 |
| T category | |
| T1-T2 | 3 |
| T3-T4 | 26 |
| Tx | 8 |
| Lymphatic spread | |
| N0 | 18 |
| N+ | 10 |
| Nx | 9 |
| Metastasis | |
| M0/Mx | 25 |
| M+ | 12 |

**Table 5.1 - Clinicopathological features of the colon carcinoma patients.**

| Variable | Number of patients |
|---|---|
| Sex | |
| Male | 10 |
| Female | 6 |
| Age at diagnosis | |
| Median age (range, years) | 68 (18-80) |
| Site of tumor | |
| Sigmoid | 9 |
| Colon transversum | 2 |
| Hepatic flexure | 1 |
| Colon ascendens | 1 |
| Caecum | 3 |
| Dysplasia | |
| Low | 10 |
| High | 4 |
| Unknown | 2 |
| Classification | |
| Tubular | 8 |
| Tubulovillous | 6 |
| Villous | 1 |
| Unknown | 1 |

**Table 5.2 - Clinicopathological features of the colon adenoma patients.**

| Variable | Number of patients |
|---|---|
| Sex | |
| Male | 16 |
| Female | 7 |
| Age at diagnosis | |
| Median age (range, years) | 62 (35-80) |
| Site of primary tumor | |
| Colon | 14 |
| Rectum | 9 |
| Synchronicity | |
| Synchronous | 15 |
| Metachronous | 8 |
| Therapy before liver resection | |
| None | 5 |
| Chemotherapy | 12 |
| Chemotherapy & bevacizumab | 4 |
| Chemotherapy & panitumab | 1 |
| Chemotherapy & bevacizumab & cetuximab | 1 |

**Table 5.3 - Clinicopathological features of the liver metastasis patients.**

### RNA-extraction, RNA-quality control and cDNA-synthesis

Total RNA was extracted with the RNeasy Plus mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. This kit contains a gDNA-elimination step to avoid gDNA contamination. After extraction, RNA quality and integrity were verified using an RNA 6000 Nano Chip Kit on the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

Only samples with adequate quality and integrity (adenoma samples 16/18, carcinoma samples 37/39, metastasis samples 23/23) were used for the RT-qPCR analysis. cDNA was synthesized from 1 µg of total RNA using Superscript® II reverse transcriptase (Invitrogen, Merelbeke, Belgium) according to the manufacturer's instructions.

**RT-qPCR**

Real-time quantitative PCR (RT-qPCR) was performed using the LC 480 Sybr Green I master kit on a LightCycler® 480 Real-Time PCR system (both from Roche Applied Science, Penzberg, Germany). Primers were designed using PrimerSelect (DNASTAR, Madison, USA) and purchased from Invitrogen. The primers were designed for gene-specific expression profiling and cover all splice variants. The sequences of the forward and reverse primers were as follows: VEGF-A 5'-TGAGTTGCCCAGGAGACCAC-3' and 5'-GAAGGGGAGCAGGAAGAGGAT-3'; VEGF-B 5'-CCGGAAGCTGCGAAGGTGACA-3' and 5'-GGGAGACAAGGGATGGCAGAAGAG-3'; VEGF-C 5'-CACGGCTTATGCAAGCAAAGA-3' and 5'-TCCTTTCCTTAGCTGACACTTGT-3'; VEGF-D 5'-GCAGCCCTAGAGAAACGTG-3' and 5'-AGGTGCTGGTGTTCATACAGAT-3'; PlGF 5'-TGCGGCGATGAGAATCTGC-3' and 5'-AGCGAACGTGCTGAGAGAAC-3'; COX2 5'-TTGCTGGAACATGGAATTACC-3' and 5'-TGCCTGCTCTGGTCAATG-3'; 5-LOX 5'-TGGCGCGGTGGATTCATAC-3' and 5'-CAGGGGAACTCGATGTAGTCC-3'; TFF3 5'-CTTGCTGTCCTCCAGCTCT-3' and 5'-CCGGTTGTTGCACTCCTT-3'; LXRalpha 5'-GGAGGTACAACCCTGGGAGT-3' and 5'-AGCAATGAGCAAGGCAAACT-3'; ApoE 5'-CTTCATGGTCTCGTCCATCAGC-3' and 5'-AAGGACGTCCTTCCCCAGGAGC-3'. All samples were assayed in triplicate. Relative expression values were calculated using the 2(-delta delta C(T)) method and were normalized against reference genes: TATA-binding protein (TBP) and succinate dehydrogenase complex subunit A (SDHA) (primers: TBP 5'-CGGCTGTTTAACTTCGCTTC-3' and 5'-CACACGCCAAGAAACAGTGA-3'; SDHA 5'-TGGGAACAAGAGGGCATCTG-3' and 5'-CCACCACTGCATCAAATTCATG-3'). For liver samples, only SDHA was used as a reference gene due to the instability of TBP as a reference gene in liver metastasis samples. In these calculations we took into account the PCR efficiency of the individual PCR reactions, calculated on the basis of linear regression as described in Ruijter et al [32]. The normalized relative expression values were scaled against the median of the

respective healthy tissue samples (median of healthy samples set to 1). The specificity of amplification was confirmed by evaluation of the melting curves.

**Statistical analysis**

Statistical analysis was performed using GraphPad Prism® software (GraphPad Software Inc., La Jolla, California, USA). For comparisons of paired samples, the Wilcoxon signed rank test was used. Comparisons of the n-fold induction between stages of progression were performed with the Kruskal-Wallis one-way ANOVA with Dunn's multiple comparison test. Comparisons of expression levels between three or four groups were performed with one-way-ANOVA with Bonferroni post-test. Significant p-values were ranked as $p<0.05$ (*), $p<0.01$ (**) and $p<0.001$ (***).

### 5.1.3 Results

**Stage-dependent induction of VEGF family members during colon cancer progression**

The mRNA expression levels of VEGF family members in samples from colon adenoma (n=16), colon carcinoma (n=37) and liver metastases (n=23) were determined by RT-qPCR. Differential expression levels were obtained by comparison with healthy tissue samples obtained from the same patients. All colon tissue samples were obtained by biopsy. Samples from liver were obtained by surgical resection.

Figure 5.1 shows the n-fold induction for each progression stage of *VEGF-A, VEGF-B, VEGF-C, VEGF-D* and *PlGF* with respect to matched healthy tissue samples. *VEGF-A* and *PlGF* were significantly ($p<0.01$ to $<0.001$) induced throughout all stages of colon cancer progression. However, whereas *VEGF-A* expression remained nearly constant in all three stages, the induction levels of *PlGF* increased concomitantly with disease progression from adenoma to carcinoma ($p<0.1$) and metastasis ($p<0.01$) (Figure 5.1A, E). Also *VEGF-B* and *VEGF-C* showed an expression increment, which however was stage-dependent, displaying significantly increased mRNA levels at the carcinoma and metastasis stages but not at the preceding adenoma stage (Figure 5.1B, C). Finally, *VEGF-D* appears to be the exception to the rule. No increased VEGF-D mRNA levels were observed in colon adenoma, carcinoma and metastasis samples (Figure 5.1D). Combined, these results indicate that already at the early stage of adenoma a distinct angiogenic signature is present, featuring the induction of *VEGF-A* and *PlGF* and further broadening towards

the expression of additional *VEGFs* upon malignant progression to carcinoma and carcinoma-derived metastases.

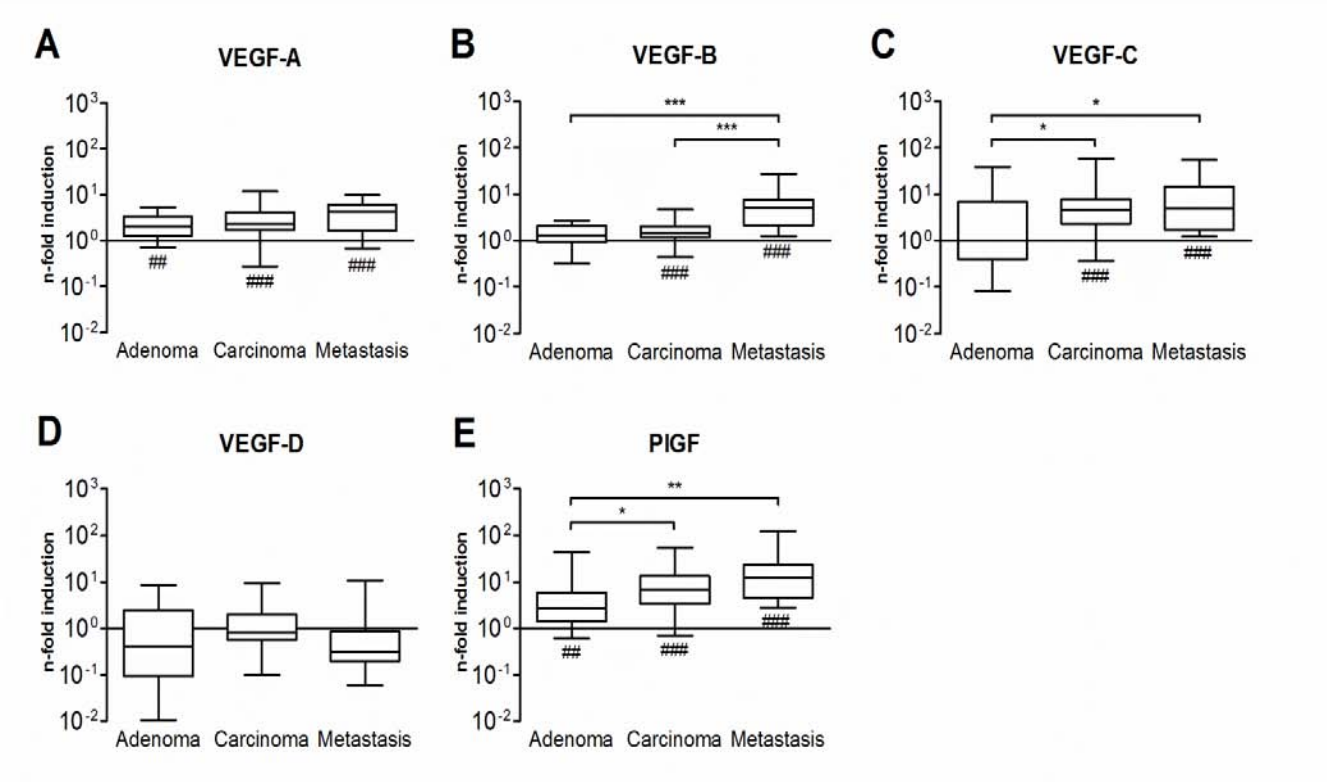


**Figure 5.1 – The induction of VEGF family members during progression of colon cancer.** n-Fold induction levels in adenoma, carcinoma and liver metastasis samples of **(A)** VEGF-A, **(B)** VEGF-B, **(C)** VEGF-C, **(D)** VEGF-D and **(E)** PlGF are shown. The n-fold induction value represents the ratio of the expression value of the diseased sample against the expression value of the paired healthy sample. The box represents the median with interquartile range and the whiskers represent minimum and maximum ratios. Significance of the induction on its own is indicated beneath the minimum whisker with ##: $p<0.01$ and ###: $p<0.001$, calculated with Wilcoxon signed rank test. Significant differences in induction between the stages is indicated above the boxes with *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$, calculated with Kruskal-Wallis one-way ANOVA with Dunn's multiple comparison test.

### Colon tissue signature of liver metastasis samples

In the above analyses, liver metastasis samples from colon carcinoma patients were compared to matched healthy tissue from liver. This implies a comparison of cancerous tissue from colon origin with healthy tissue originating from a distinctly different organ. We therefore verified to what extent liver metastasis samples retained the characteristics of their tissue of origin. Hereto, we determined the mRNA expression levels of tissue markers specific for colon and liver on a subset of colon carcinoma (n=10) and liver metastasis samples (n=10). As shown in Figure 5.2B, the *Trefoil Factor 3* (*TFF3*) colon marker was equally expressed in liver metastasis samples and

healthy colon samples as opposed to the absence of *TFF3* expression in healthy liver samples. This differential expression pattern confirms the colonic nature of the liver metastases. However, liver metastasis samples also showed a liver-specific gene signature. This is apparent from the significantly higher expression levels in liver metastasis samples of the liver markers *Liver X Receptor alpha* (LXRalpha) and *Apolipoprotein E* (ApoE), showing values similar to (*LXRalpha)* or somewhat below (*ApoE)* healthy liver (Figure 5.2C-D). The reference gene *SDHA* was equally expressed in all sample groups, suggesting that the normalization on the samples was not influenced by the tissue (Figure 5.2A).

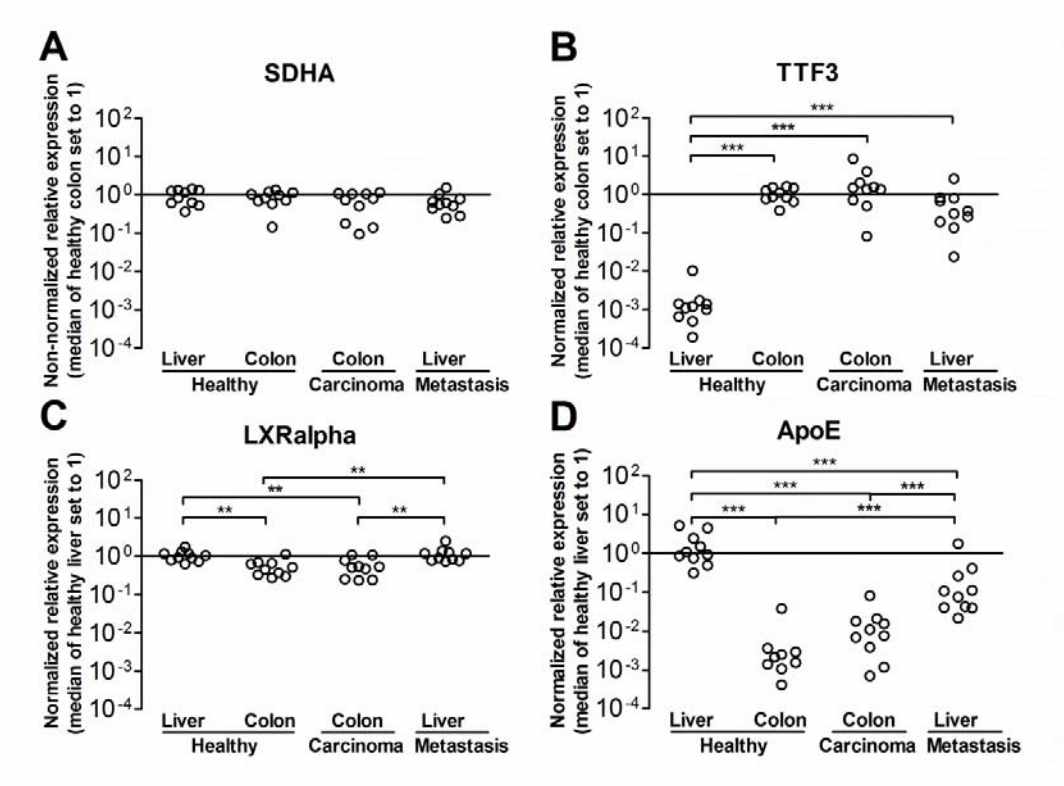


**Figure 5.2 – Comparison of colon and liver markers.** Relative mRNA expression levels of the reference gene **(A)** SDHA, the colon marker **(B)** TTF3 and the liver markers **(C)** LXRalpha and **(D)** ApoE are shown for healthy liver and colon, colon carcinoma and liver metastasis samples. Expression levels were normalized (except SDHA) against the reference gene SDHA and were scaled against the median of the biopsy samples (median set to 1). Expression data are depicted as scatter plots of the values obtained for each individual sample. Significant differences are indicated by **: $p<0.01$ and ***: $p<0.001$, calculated with Kruskal-Wallis one-way ANOVA with Dunn's multiple comparison test.

Because of the colonic signature of liver metastasis samples and its contamination with a liver signature, we next verified in the same subset of the samples to what extent n-fold induction levels of VEGF family members in liver metastasis samples may be biased by the tissue origin –

liver or colon – of the healthy tissue calibrator. Except for *VEGF-B* showing similar n-fold induction values irrespective of the tissue origin of the healthy tissue calibrator (Figure 5.3B), all VEGF family members showed significantly higher levels of overexpression when liver metastasis samples were compared with healthy colon instead of healthy liver (Figure 5.3A, C-E). This analysis indicates that at the stage of metastasis the expression increments may be even more pronounced than indicated on the basis of using healthy liver samples as calibrator (see also supplemental Figure S5.1).

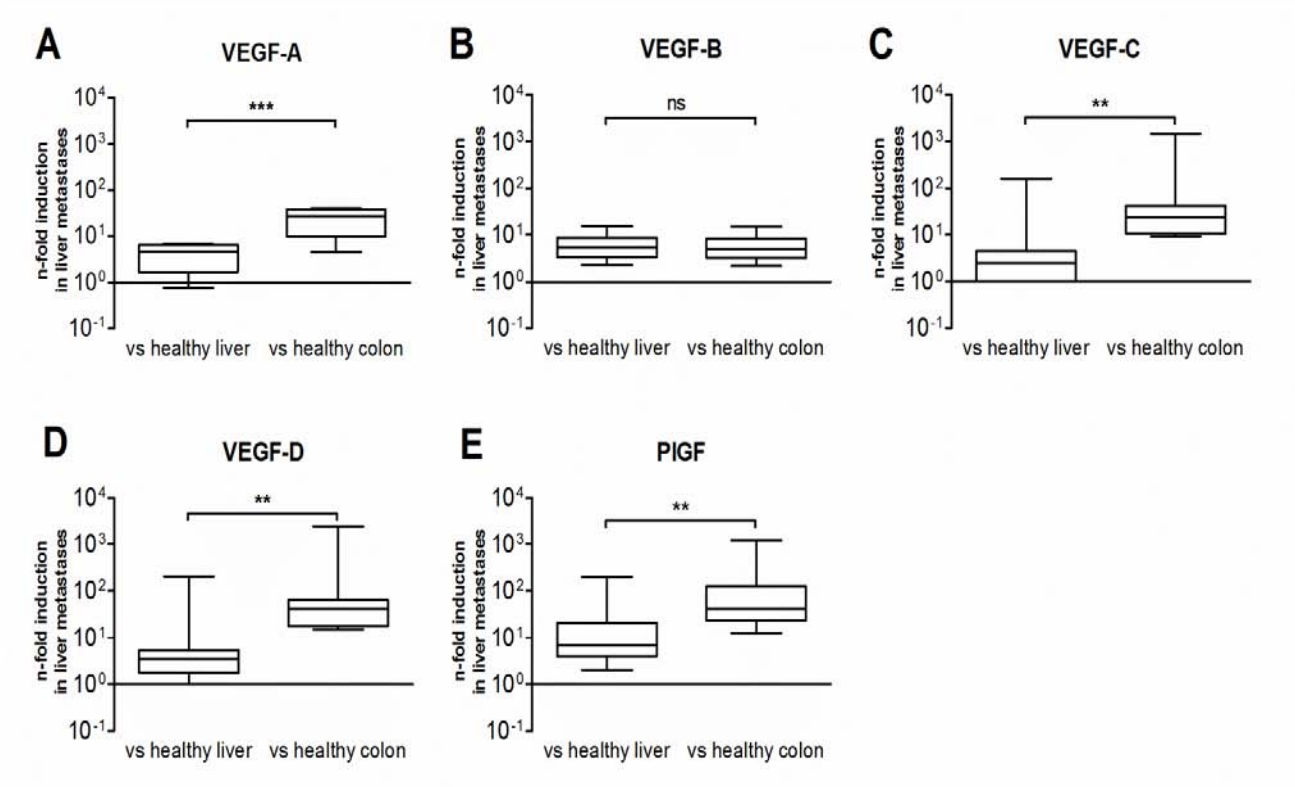


**Figure 5.3 – Impact of the tissue origin – liver or colon – of the healthy tissue calibrator for liver metastasis samples.** n-Fold induction levels in liver metastasis versus healthy liver and healthy colon of **(A)** VEGF-A, **(B)** VEGF-B, **(C)** VEGF-C, **(D)** VEGF-D and **(E)** PlGF are shown. The n-fold induction value represents the ratio of the expression value of each metastasis sample against the median expression value of the healthy liver or colon samples. The box represents the median with interquartile range and the whiskers represent minimum and maximum ratios. Significant differences in induction between the stages is indicated above the boxes with ns: not significant; **: $p<0.01$; ***: $p<0.001$, calculated with Mann-Whitney U test.

## Individual patient signatures reveal a cumulative angiogenic expression increment with malignant progression

*VEGF* expression signatures from individual colon adenoma, colon carcinoma and liver metastasis samples were determined to verify to what extent the progression-associated

broadening of angiogenic gene expression we observed at the population level can be observed also at the level of the individual patient. For the analysis of liver metastases, a conservative approach was followed based on the usage of healthy samples from liver as a calibrator. The cut-off for defining overexpression was set on the median value + (2.58 x SD) of the corresponding healthy tissue cohort.

As illustrated in Figure 5.4, overexpression of a single *VEGF* family member is the predominating pattern at the adenoma stage (37%), closely followed by absence of overexpression (25%) and overexpression of two *VEGF* family members (25%). At the colon carcinoma stage, a pronounced shift towards the right of the axis is observed, illustrating co-overexpression of three (27%) up to four (40,50%) *VEGF* family members. Metastasis samples confirm this shift towards overexpression of multiple VEGF genes and show an even more pronounced polarization towards the concomitant overexpression of four (52%) VEGF genes.

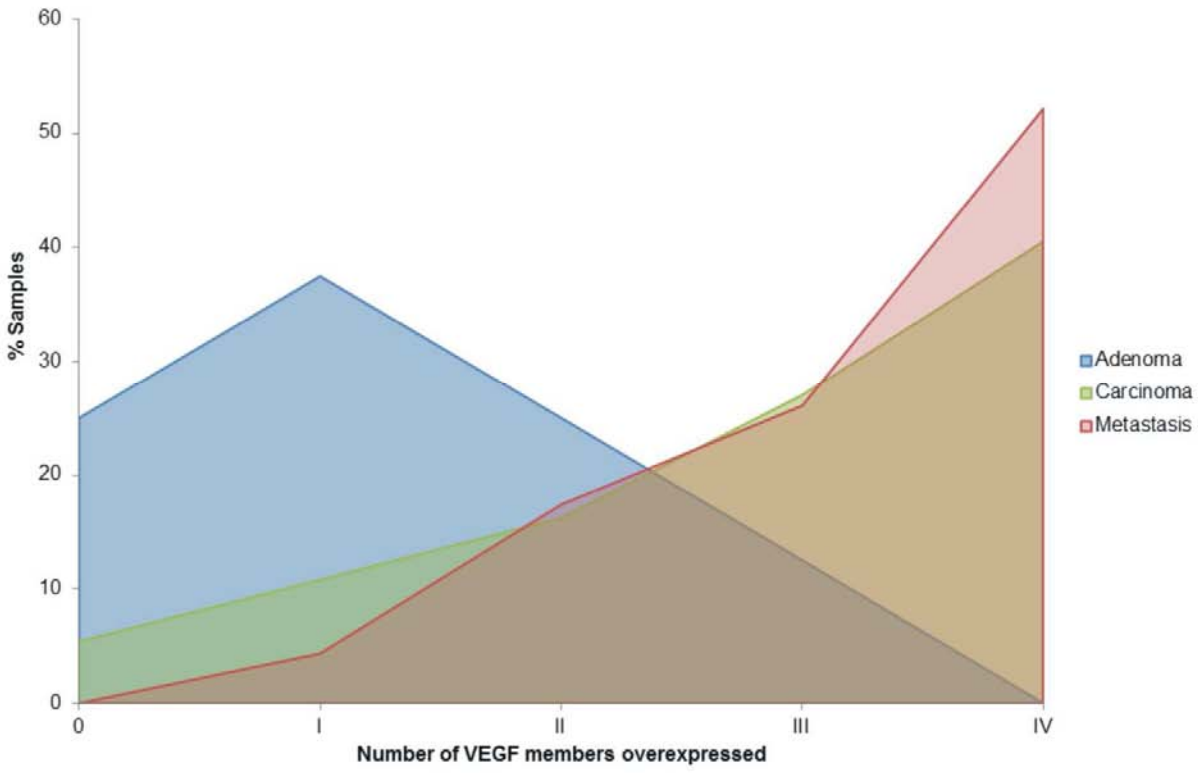


**Figure 5.4 – Angiogenic shifts in individual patient signatures during colon carcinoma progression.** The graph shows the percentages of samples which overexpressed none up to four VEGF family members. The cut-off for overexpression was based on the median of the healthy samples for each population group +2.58SD.

### 5.1.4 Discussion

Studies on the angiogenesis promoting function in colon cancer of VEGFs and their potential for angiogenic therapy are strongly focused towards VEGF-A. However, also VEGF-B, VEGF-C, VEGF-D and especially PlGF are emerging as candidate angiogenic factors promoting carcinogenesis [14, 17, 27]. Yet it remains unclear to what extent the different VEGFs are (co-)expressed in human colon cancer and evolve in the process of malignant transformation and progression. Despite numerous reports in literature, the often scattered and contradictory nature of the data makes it hard to obtain a global picture on VEGF angiogenic expression in colon cancer and during colon carcinoma progression. Here, we analyzed in a systematic way the mRNA expression levels of all individual VEGFs in colon carcinoma samples in comparison with colon adenoma and liver metastasis samples.

Already at the early stage of colon adenoma, a distinctive angiogenic profile featuring a strong induction of *VEGF-A* and *PlGF* was apparent. This profile persisted in carcinoma and liver metastasis samples. Increased *VEGF-A* expression in adenomas that persists in carcinomas has been reported by several research groups [27, 33, 34] although others failed to detect VEGF-A mRNA expression at the adenoma [29, 35] or carcinoma stage [18]. Our results clearly point towards increased VEGF-A mRNA levels from the start of the adenoma-carcinoma sequence and persisting throughout the sequence. Furthermore, our expression analysis identifies *PlGF* as a second angiogenic factor expressed early on in colon cancer progression. The expression of *PlGF* already in colon adenomas and even more pronounced in colon carcinomas and liver metastases identifies PlGF as a potential target for angiogenic therapy and a possible cause of angiogenic escape upon VEGF-A-targeting therapy. Finally the expression patterns of *VEGF-B* and *VEGF-C* – lacking in colon adenoma but increased in colon carcinoma and liver metastasis – identify both angiogenic factors as potential markers for malignant progression. Again, these results are in line with previous reports by Rmali and Wang [18, 25] but contradict others reporting unchanged VEGF-B mRNA levels during neoplastic progression of colon tissue [22, 23, 26] or increased *VEGF-B* expression only in the adenoma stage [27]. Methodological differences including less quantitative mRNA-measuring techniques such as semi-quantitative RT-PCR [23, 29], Northern blot [26] or RT-PCR [27, 35] likely account for some of the contradictory observations. Furthermore, most studies included rectal samples [18, 22, 23, 27, 29, 35]. Because radiotherapy

prior to surgery is standard procedure in rectal cancer, we excluded such patients from our study [26]. A third confounding factor may in fact be the clinical procedure applied for obtaining tissue samples. Whereas several studies used samples obtained by surgical resection [18, 22, 26, 27, 35], we used in this study colon samples obtained by colonoscopic biopsy. In a recent study we showed that biopsy samples provide a more accurate report on VEGF family mRNA levels than samples obtained by surgical resection since sampling-induced hypoxia in resection samples affects the mRNA expression of VEGF family members [36].

The mRNA-analysis of liver metastases necessitates an extra consideration: do liver metastases have to be compared with healthy liver – the tissue where they were found – or with healthy colon – the tissue they originate from? To address this question, we examined in a subset of samples to what extent liver metastasis samples retained the characteristics of their tissue of origin or gained characteristics of their new environment. The expression pattern confirmed the colonic nature of the liver metastases, showing mRNA levels of the colon marker TTF3 similar to healthy colon. Yet, liver metastasis samples also scored positive for the liver tissue markers *LXRalpha* and *ApoE*. Likely, this additional liver tissue signature reflects the presence in the metastasis samples of residential liver cells. Importantly, the ectopic origin of the liver metastases did not affect the conclusions from our comparative VEGF gene expression analysis. Thus VEGF family members showed similar (VEGF-B) or even higher (VEGF-A, -C, -D and PlGF) higher induction values when liver metastasis samples were compared with healthy colon instead of healthy liver. This analysis therefore indicates that the angiogenic profile of colon carcinoma is conserved when metastasizing to other body tissues. This conclusion is further supported when looking at *VEGF* expression signatures from individual samples. Here, a strong shift towards the co-expression of multiple *VEGFs* is apparent upon progression from adenoma to carcinoma. In contrast, the VEGF signature of individual carcinoma samples is near indistinguishable from that of liver metastasis samples. From a therapeutic angle, the apparent conservation of angiogenic profiles indicates angiogenic therapy will equally target primary colon carcinoma and its secondary metastases, provided the therapy is individualized to the patient's VEGF signature. Furthermore, the occurrence of co-expressed *VEGFs* in individual samples supports the notion put forward by Fan and colleagues using colorectal cell lines that co-expression of multiple VEGF family members is at the basis of angiogenic escape to VEGF-A neutralizing treatments [11].

In conclusion, this comprehensive and systematic analysis of *VEGFs* mRNA expression reveals the complex nature of angiogenic gene expression already at the stage of adenoma and its further deviation towards co-expression of multiple angiogenic genes upon progression to carcinoma and liver metastasis. Furthermore, our expression analysis identifies *VEGF-B* and *VEGF-C* as angiogenic genes who are upregulated only in carcinoma and metastasis stages whereas *PlGF* and *VEGF-A* are present already at the stage of adenoma. Unraveling the molecular pathway(s) at the basis of the manifold *VEGF* expression may help to resolve molecular processes controlling angiogenesis in cancer.

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## SUPPLEMENTAL FIGURE

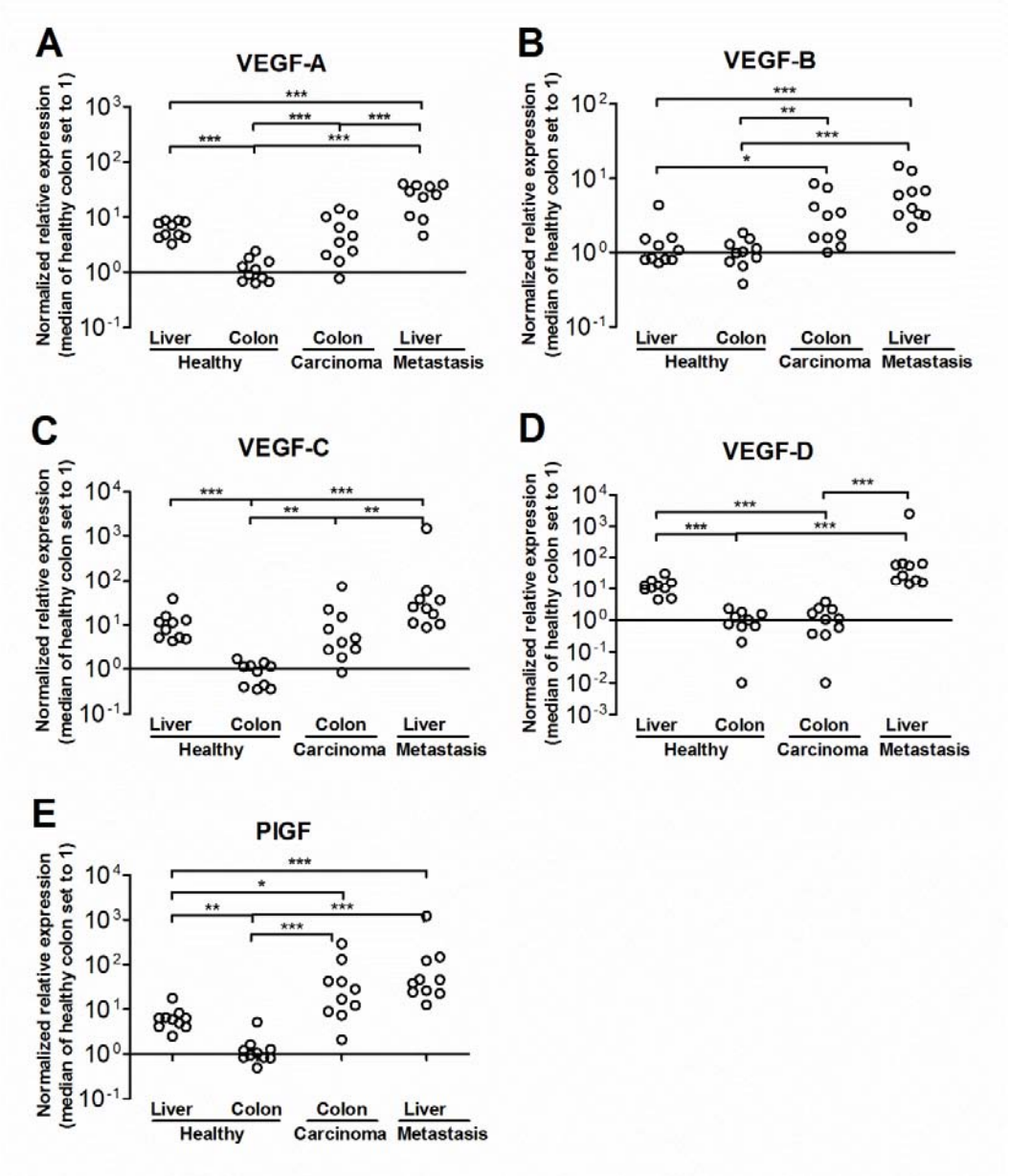


**Figure S5.1 – Comparison of VEGF family members in colon and liver.** Relative mRNA expression levels of **(A)** VEGF-A, **(B)** VEGF-B **(C)** VEGF-C, **(D)** VEGF-D and **(E)** PlGF are shown for healthy liver and colon, colon carcinoma and liver metastasis samples. Expression levels were normalized against the reference gene SDHA and were scaled against the median of the biopsy samples (median set to 1). Expression data are depicted as scatter plots of the values obtained for each individual sample. Significant differences are indicated by **: $p<0.01$ and ***: $p<0.001$, calculated with Kruskal-Wallis one-way ANOVA with Dunn's multiple comparison test.

## 5.2 Additional data

### 5.2.1 VEGF-A, VEGF-C and PlGF serum levels in colon adenoma and carcinoma patients

The observed shift in VEGFs mRNA signatures between colon adenoma and carcinoma tissue samples gives rise to the question if similar signatures exist also at the protein level. Therefore the protein levels of VEGF-A, VEGF-C and PlGF were measured in serum from healthy individuals who underwent colonoscopy (n= 31) and from patients with either colon adenoma (n= 21) or colon carcinoma (n= 30). The analysis was performed with a quantitative Luminex®-based multiplex immunoassay using a commercially available kit (MILLIPLEX®MAP Human Angiogenesis/Growth Factor Magnetic Bead Panel kit, Millipore, Overijse, Belgium). Because such analysis was not possible for VEGF-B due to a lack of commercially available and sensitive quantification assays, VEGF-B was omitted from this analysis. Also VEGF-D was not included on the basis of its inert mRNA expression in the different stages of colon cancer (see 5.1). Surprisingly, as shown in figure 5.5, VEGF-A, VEGF-C and PlGF serum levels were similar in the healthy, adenoma and carcinoma serum samples.

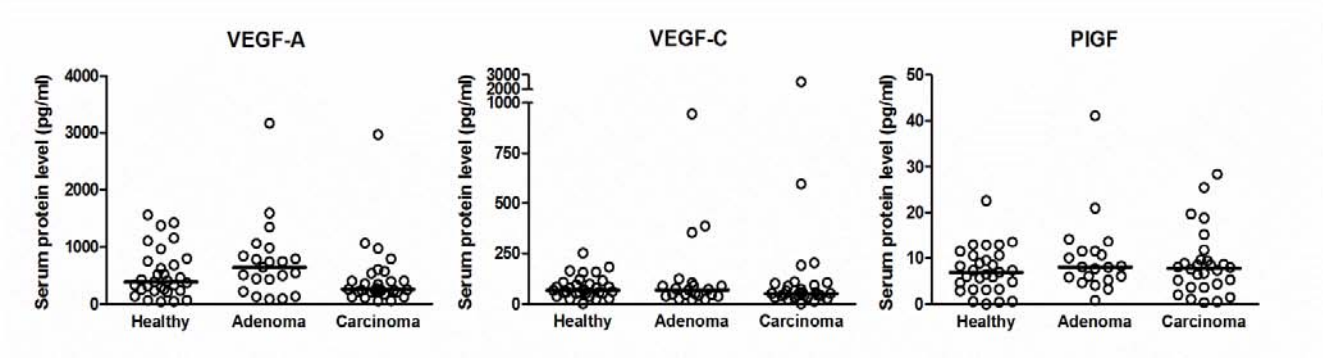


**Figure 5.5 - Protein levels of VEGF-A, VEGF-C and PlGF serum from healthy persons and patients with colon adenoma or colon carcinoma.** The serum levels of VEGF-A, VEGF-C and PlGF are shown as measured by a quantitative Luminex®-based multiplex analysis in serum samples from healthy individuals and patients with colon adenoma or carcinoma. Protein levels are depicted as scatter plots of the values obtained for each individual sample. Significant differences were calculated with Kruskal-Wallis one-way ANOVA with Dunn's multiple comparison test.

In order to confirm these results, an ELISA was performed for PlGF (Quantikine® ELISA Human PlGF Immunoassay, R&D Systems, Abingdon, UK). In contrast to the similar PlGF serum levels obtained with the multiplex immunoassay, significant differences were observed between serum from healthy individuals and carcinoma patients (Figure 5.6).

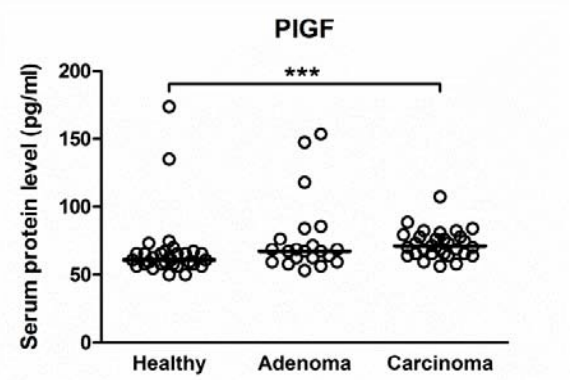


**Figure 5.6 - Serum levels of PlGF in adenoma and carcinoma serum samples obtained by ELISA.** Serum levels of PlGF are shown as measured by ELISA in serum samples from healthy individuals and patients with colon adenoma or carcinoma. Protein levels are depicted as scatter plots of the values obtained for each individual sample. Significant differences are indicated by ***: $p<0.001$, calculated with Kruskal-Wallis one-way ANOVA with Dunn's multiple comparison test.

Thus, two commercial kits produced opposite results on PlGF serum levels; no differences in PlGF-levels using the Luminex®-based immunoassay as opposed to significant differences between healthy and carcinoma serum samples using ELISA. Yet, the ELISA-based results agree well with previously published reports on increased serum levels of PlGF in colon cancer patients [1, 2]. On this basis we presume that the results from the ELISA-assay are authentic, indicating that at the stage of colon carcinoma increased PlGF serum levels may be present in the blood in line with the increased PlGF mRNA levels we observed by RT-qPCR in carcinoma biopsies. At the stage of colon adenoma, the increment in PlGF serum levels is not significant with Kruskal-Wallis one-way ANOVA. However, when comparing the serum levels of the healthy individuals and adenoma patients by means of a Mann-Whitney U test, a significant increment ($p<0.05$) in serum PlGF levels is observed in the adenoma patients. Yet, expansion to a larger patient cohort is indicated to obtain solid conclusions on PlGF serum levels at the stage of colon adenoma.

As for VEGF-A and VEGF-C serum levels, no significant differences were observed between healthy, colon adenoma and carcinoma samples using the Luminex®-based immunoassay. This is in contrast with previous studies reporting increased levels of serum VEGF-A and/or VEGF-C in colorectal cancer patients [3-8]. Although this discrepancy might be due to the detection assay used, also other features may underlie this discrepancy with literature. Because VEGF-A is also released by activated platelets during blood clotting, several studies have reported that the levels in serum do not necessarily reflect the actual level of circulating VEGF-A but rather platelet degranulation during clotting [9, 10]. Therefore, plasma VEGF-A levels rather than serum levels

may better reflect the ongoing angiogenic activity because less platelet activation is to be expected in plasma [9, 11]. In line herewith, plasma levels of VEGF-A have been ascribed a prognostic value in colorectal cancer [9, 12, 13]. Other studies however reported that plasma VEGF-A levels were not increased in colorectal cancer patients and showed no association with clinicopathological variables [14]. Possibly, VEGF-A released by platelets may also contribute to increased VEGF-A levels in plasma, although to a far lesser extent than in serum [15]. Also VEGF-C is released by platelets during clotting and therefore also VEGF-C serum levels may be confounded with platelet-derived VEGF-C [16, 17]. Clearly, the debate on the ideal type of blood sample for measuring circulating VEGF-A and VEGF-C is still ongoing. It is proposed when using serum to include platelet counts in the readout, thus allowing for normalization of platelet numbers between the samples and a better comparison between groups [18].

### 5.2.2 Association between VEGF family members and clinicopathological variables

In order to verify whether any of the overexpressed VEGF family members are associated with clinicopathological features of colon adenoma, carcinoma and liver metastasis samples, multiple comparisons were performed between the *VEGFs* expression profiles and clinicopathological variables. *VEGF-A, VEGF-B* and *VEGF-C* expression levels correlated significantly with the histology (tubular or villous/tubulovillous) of colon adenoma samples (Table 5.4, Figure 5.7). *VEGF-A* expression levels were significantly higher in villous and tubulovillous adenoma than in tubular adenoma, whereas the expression levels of *VEGF-B* and *VEGF-C* were higher in tubular adenoma. However, since both *VEGF-B* and *VEGF-C* were not significantly induced in colon adenoma, this correlation probably is coincidental. Finally, no correlations were found with age, gender, localization in the colon or the grade of dysplasia in adenoma (Table 5.4).

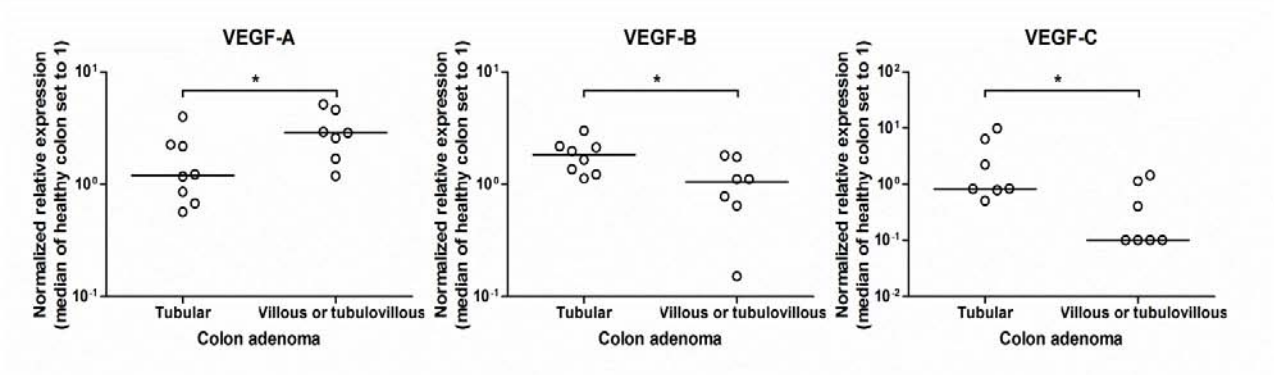


**Figure 5.7 - Comparison of expression levels in tubular adenoma versus villous or tubulovillous adenoma** for VEGF-A, VEGF-B and VEGF-C with Mann-Whitney test. *: $p<0.05$

In colon carcinoma and liver metastasis, neither of the VEGFs correlated with general variables as age or gender or localization of the primary colon carcinoma (Table 5.5 and 5.6). Only VEGF-A showed significance when comparing carcinoma samples from different locations in the colon, although Dunn's multiple comparison test did not find specific significant differences between the colonic regions (Table 5.5, Figure 5.8).

Also disease specific variables – differentiation grade or Dukes classification, therapy before resection of liver metastases – showed no correlation with expression levels of *VEGFs*. On the other hand, overexpression of *VEGF-A* was significantly higher in metachronous liver metastases as compared to synchronous liver metastases (Table 5.6, Figure 5.9).

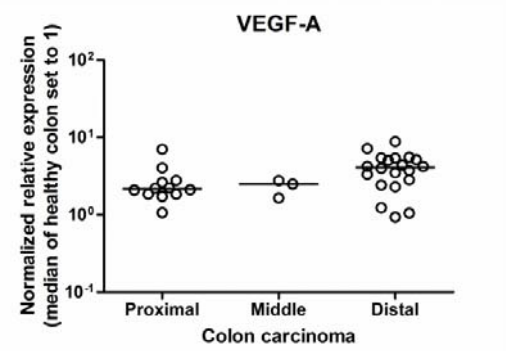


**Figure 5.8 - Comparison of VEGF-A expression levels in carcinoma from proximal, middle or distal regions in the colon** with Kruskal Wallis test. The difference between the medians is statistically significant ($p<0.05$), but Dunn's multiple comparison test detects no specific significant difference between two groups.

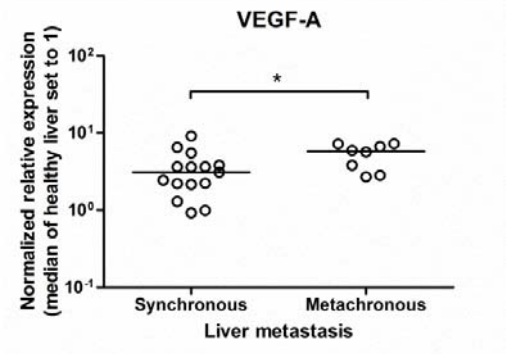


**Figure 5.9 - Comparison of VEGF-A expression levels in synchronous versus metachronous liver metastases** with Mann-Whitney test. *: $p<0.05$

Overall, few significant correlations were found between the expression levels of VEGF family members and clinicopathological variables in either of the three stages of colon cancer. Only *VEGF-A* was found to correlate with villous and tubulovillous adenoma, which have a higher intrinsic risk of malignant transformation than tubular adenoma. However, since *VEGF-A* is not correlated with a high degree of dysplasia in adenoma – another adenomatous feature with an increased risk of malignant transformation – it is hard to state that high levels of *VEGF-A* are associated with an increased risk of malignant transformation. High expression levels of *VEGF-A* were also correlated with metachronous liver metastasis, which may indicate an association of higher VEGF-A mRNA levels with recurrence of metastasis. This agrees with a previous report by Min and colleagues, who found that high VEGF-A serum levels are associated with a high risk of metachronous liver metastasis and hepatic recurrence following the resection of synchronous liver metastasis [8]. In addition, pre-operative VEGF-A protein levels in serum and carcinoma samples from colorectal cancer patients have been demonstrated to predict recurrence and development of metastases following curative surgery of colorectal carcinoma [19-21].

| | | | VEGF-A | | | VEGF-B | | | VEGF-C | | | PlGF | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Colon adenoma** | | n | ME | SD | p-value | ME | SD | p-value | ME | SD | p-value | ME | SD | p-value |
| **Age**[1] | <70 years | 10 | 1.59 | 1.49 | 0.7925 | 1.51 | 0.67 | 1.000 | 1.14 | 8.66 | 0.3530 | 2.21 | 6.65 | 0.1806 |
| | ≥70 years | 6 | 2.38 | 1.34 | | 1.12 | 0.82 | | 0.65 | 0.80 | | 3.42 | 5.70 | |
| **Gender**[2] | Male | 10 | 1.94 | 1.43 | 0.7925 | 1.55 | 0.70 | 0.2198 | 0.81 | 2.88 | 0.9130 | 3.14 | 7.42 | 0.3676 |
| | Female | 6 | 2.17 | 1.47 | | 1.11 | 0.69 | | 0.82 | 10.96 | | 2.45 | 2.07 | |
| **Localization**[3] | Proximal | 4 | 1.94 | 1.91 | 0.6768 | 1.55 | 0.98 | 0.1489 | 1.14 | 0.90 | 0.2417 | 2.20 | 1.18 | 0.3759 |
| | Middle | 3 | 1.17 | 1.84 | | 1.97 | 0.26 | | 6.41 | 4.55 | | 17.2 | 10.96 | |
| | Distal | 9 | 2.26 | 1.18 | | 1.11 | 0.58 | | 0.50 | 9.09 | | 2.85 | 1.85 | |
| **Dysplasia**[4] | Low | 10 | 1.46 | 1.53 | 0.3736 | 1.70 | 0.67 | 0.3037 | 1.14 | 8.62 | 0.2273 | 2.68 | 7.53 | 0.6354 |
| | High | 4 | 2.42 | 1.42 | | 1.17 | 0.43 | | 0.59 | 0.45 | | 3.97 | 1.28 | |
| **Histology**[5] | Tubular | 8 | 1.20 | 1.16 | 0.0401* | 1.81 | 0.62 | 0.0205* | 1.52 | 9.38 | 0.0228* | 3.14 | 8.07 | 0.3969 |
| | Villous/ Tubulo-villous | 7 | 2.87 | 1.43 | | 1.11 | 0.95 | | 0.10 | 0.57 | | 2.85 | 1.50 | |

ME: median; SD: standard deviation

[1] Comparison of expression levels in patients younger than 70 years versus patients of 70 years or older with Mann-Whitney test.

[2] Comparison of expression levels in male versus female patients with Mann-Whitney test.

[3] Comparison of expression levels in samples from different adenoma sites with Kruskal Wallis test.

[4] Comparison of expression levels in samples with low grade versus high grade dysplasia with Mann-Whitney test.

[5] Comparison of expression levels in samples from tubular versus villous/tubulovillous adenoma with Mann-Whitney test. *: $p<0.05$

**Table 5.4 – Comparison of the expression of COX2, 5-LOX and VEGF family members with clinicopathological features in colon adenoma.**

| | | | VEGF-A | | | VEGF-B | | | VEGF-C | | | PlGF | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Colon carcinoma** | | n | ME | SD | p-value | ME | SD | p-value | ME | SD | p-value | ME | SD | p-value |
| **Age**[1] | <70 years | 18 | 2.80 | 2.33 | 0.8912 | 1.64 | 0.78 | 0.8673 | 3.71 | 5.68 | 0.8197 | 8.49 | 8.44 | 0.8434 |
| | ≥70 years | 19 | 2.63 | 1.45 | | 1.40 | 0.91 | | 3.12 | 10.55 | | 7.44 | 19.14 | |
| **Gender**[2] | Male | 21 | 3.32 | 1.65 | 0.8708 | 1.72 | 0.76 | 0.5643 | 3.02 | 10.17 | 0.3831 | 7.44 | 18.49 | 0.9176 |
| | Female | 16 | 2.36 | 2.25 | | 1.37 | 0.95 | | 3.71 | 5.77 | | 7.24 | 7.83 | |
| **Localization**[3] | Proximal | 12 | 2.15 | 1.55 | 0.0401* | 1.83 | 1.08 | 0.4058 | 3.91 | 13.74 | 0.3468 | 12.99 | 22.59 | 0.6849 |
| | Middle | 3 | 2.48 | 0.58 | | 1.24 | 0.91 | | 1.91 | 2.45 | | 4.34 | 5.96 | |
| | Distal | 21 | 4.30 | 1.90 | | 1.50 | 0.64 | | 3.39 | 1.60 | | 7.69 | 4.72 | |
| **Differentiation**[4] | Low | 12 | 2.46 | 1.19 | 0.3662 | 1.46 | 0.89 | 0.8589 | 3.07 | 14.09 | 0.5805 | 5.58 | 23.04 | 0.9056 |
| | Moderate | 18 | 3.72 | 1.96 | | 1.72 | 0.64 | | 3.66 | 2.97 | | 7.94 | 8.39 | |
| | High | 3 | 3.50 | 3.08 | | 1.59 | 0.34 | | 5.49 | 0.31 | | 14.40 | 10.52 | |
| **Classification**[5] | Dukes ‘A | 3 | 4.21 | 3.12 | 0.7199 | 1.13 | 0.41 | 0.6361 | 3.77 | 1.79 | 0.5012 | 7.44 | 6.78 | 0.7614 |
| | Dukes’ B | 15 | 3.32 | 1.49 | | 1.69 | 0.76 | | 4.45 | 12.55 | | 12.02 | 21.25 | |
| | Dukes’ C | 7 | 2.23 | 1.40 | | 1.40 | 0.51 | | 3.02 | 3.82 | | 6.53 | 5.56 | |
| | Dukes’ D | 12 | 2.56 | 2.42 | | 1.92 | 1.07 | | 3.56 | 1.65 | | 4.48 | 6.98 | |

ME: median; SD: standard deviation

[1] Comparison of expression levels in patients younger than 70 years versus patients of 70 years or older with Mann-Whitney test.

[2] Comparison of expression levels in male versus female patients with Mann-Whitney test.

[3] Comparison of expression levels in samples from different carcinoma sites with Kruskal Wallis test. *: $p<0.05$

[4] Comparison of expression levels in colon carcinoma samples with different differentiation grades with Kruskal Wallis test.

[5] Comparison of expression levels in colon carcinoma with either of Dukes’ A, B, C or D classification with Kruskal Wallis test.

**Table 5.5 – Comparison of the expression of COX2. 5-LOX and VEGF family members with clinicopathological features in colon carcinoma.**

| | | | VEGF-A | | | VEGF-B | | | VEGF-C | | | PlGF | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Liver metastasis** | | n | ME | SD | p-value | ME | SD | p-value | ME | SD | p-value | ME | SD | p-value |
| **Age**[1] | <70 years | 17 | 3.63 | 2.24 | 0.8611 | 4.59 | 2.92 | 0.8611 | 4.25 | 5.11 | 0.4207 | 10.82 | 29.10 | 0.7002 |
| | ≥70 years | 6 | 3.13 | 2.52 | | 5.11 | 4.61 | | 6.27 | 20.64 | | 15.26 | 30.51 | |
| **Gender**[2] | Male | 16 | 4.67 | 2.45 | 0.1328 | 5.19 | 3.51 | 0.1328 | 4.94 | 6.73 | 0.4423 | 10.59 | 25.03 | 0.9202 |
| | Female | 7 | 2.82 | 1.03 | | 2.76 | 2.66 | | 3.19 | 19.47 | | 11.50 | 38.45 | |
| **Localization**[3] | Rectum | 9 | 3.63 | 2.05 | 0.8598 | 5.80 | 2.88 | 0.4858 | 4.99 | 16.45 | 0.2997 | 11.38 | 41.48 | 0.4762 |
| | Sigmoid | 11 | 3.65 | 2.57 | | 4.05 | 3.73 | | 3.19 | 8.25 | | 11.37 | 14.95 | |
| | Other colon | 3 | 2.45 | 2.42 | | 2.33 | 4.06 | | 3.59 | 0.92 | | 5.35 | 7.71 | |
| **Synchronous/ metachronous**[4] | Synchr. | 15 | 3.09 | 2.22 | 0.0359* | 4.48 | 3.73 | 0.8213 | 3.96 | 7.07 | 0.5397 | 11.37 | 30.93 | 0.9743 |
| | Metachr. | 8 | 5.81 | 1.90 | | 4.81 | 2.62 | | 6.74 | 17.60 | | 10.87 | 26.20 | |
| **Therapy before liver resection**[5] | None | 5 | 3.81 | 2.50 | 0.7805 | 6.41 | 4.90 | 0.4825 | 4.99 | 22.35 | 0.5013 | 7.55 | 13.42 | 0.4607 |
| | Chemo-therapy | 12 | 3.63 | 1.95 | | 4.76 | 2.77 | | 4.11 | 5.71 | | 14.43 | 36.61 | |
| | Chemo-therapy + biological | 6 | 4.32 | 2.84 | | 3.46 | 2.83 | | 3.14 | 3.53 | | 10.59 | 6.72 | |

ME: median; SD: standard deviation; Synchr.: synchronous; Meta.: metachronous

[1] Comparison of expression levels in patients younger than 70 years versus patients of 70 years or older with Mann-Whitney test.

[2] Comparison of expression levels in male versus female patients with Mann-Whitney test.

[3] Comparison of expression levels in metastasis with different primary tumor sites with Kruskal Wallis test.

[4] Comparison of expression levels in metachronous versus synchronous liver metastasis with Mann-Whitney test. *: $p<0.05$

[5] Comparison of expression levels between different therapy conditions prior to resection with Kruskal Wallis test.

**Table 5.6 – Comparison of the expression of COX2. 5-LOX and VEGF family members with clinicopathological features in liver metastasis.**

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# Chapter 6

# Eicosanoids in the driver's seat for VEGFs expression profiles?

**Contributions**

This study was conceived and designed by Sarah Pringels, Nancy Van Damme, Johan Grooten and Marc Peeters.

All technical experiments were performed by Sarah Pringels.

The acquisition of patient data and clinical samples was coordinated by Nancy Van Damme and Karen Geboes.

Expert advice and support on RT-qPCR expression analysis were provided by Bram De Craene.

The invasion assays were guided and supported by Olivier De Wever and Marc Bracke.

## 6.1 Intertwined inflammatory and angiogenic shift during malignant transformation of colon adenoma to colon carcinoma

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### Abstract

Shifts between colon adenoma and colon carcinoma stages in the expression profiles of vascular endothelial growth factor (VEGF) family members have been reported before. Because eicosanoids are well known inducers of VEGFs and angiogenesis, we performed a correlated mRNA expression analysis of enzymes involved in eicosanoid synthesis and VEGF family members. Hereto, a comparative RT-qPCR analysis was performed on colon adenoma and carcinoma samples to measure mRNA expression levels of VEGF-A, VEGF-B, VEGF-C and PlGF as well as of genes encoding the eicosanoid enzymes cyclooxygenase 2 (COX2) and 5-lipoxygenase (5-LOX). The observed eicosanoid enzyme expression levels revealed differential profiles with a *5-LOX* signature present in adenoma samples and a *COX2* signature in carcinoma samples. Dual expression of *COX2* and *5-LOX* was associated with high grade dysplastic and villous adenoma samples and correlated in adenoma and carcinoma samples with the co-expression of multiple VEGF family members. This correlated mRNA expression analysis thus demonstrates the occurrence of distinctive *5-LOX* and *COX2* expression profiles for colon adenoma and carcinoma stages and their overexpression in these samples showing cumulative expression of multiple VEGF family members. In addition, dual expression of *COX2* and *5-LOX* in adenoma may indicate an increased likelihood of malignant progression to carcinoma.

### 6.1.1 Introduction

Eicosanoids comprise a set of lipid inflammatory mediators derived from arachidonic acid metabolism and play a pivotal role in cancer development and progression [1, 2]. Especially prostanoids, generated through cyclooxygenase (COX) 1 and COX2, and leukotriene-products of 5-lipoxygenase (5-LOX) are inflammatory mediators promoting in addition to inflammation also angiogenesis through the induction of vascular endothelial growth factors (VEGF) [3-5]. Prostaglandin (PG) $E_2$ has been shown to stimulate the translocation of HIF-1 to the nucleus and hereby to indirectly promote the expression of VEGF-A [6, 7]. Also the expression of VEGF-C is induced by COX2 via the PGE2-pathway [8]. From the leukotrienes (LT) generated through 5-LOX, especially $LTB_4$ and $LTD_4$ have been shown to induce expression of VEGF-A [5, 9].

We have observed a substantial shift in expression signatures of VEGF family members between adenoma and carcinoma stages, with *PlGF* and *VEGF-A* overexpressed already at the adenoma stage and supplemented by *VEGF-B* and *VEGF-C* in the carcinoma stage [10]. Moreover, individual patient samples showed an increasing number of overexpressed *VEGFs* when comparing adenoma (0 to 2 *VEGFs*) with carcinoma (3 to 4 *VEGFs*) samples [10]. Because eicosanoids are well-known inducers of VEGFs and angiogenesis, we performed a correlation study to verify if the stage-dependent angiogenic signatures observed in colon cancer coincide with mRNA expression signatures of COX2 and 5-LOX. Although the expression of COX2 has already been reported to correlate with VEGF-A and VEGF-C expression in several types of cancer [3, 11-17], up to now no systematical expression analysis of the VEGF family members and eicosanoid enzymes has been reported for colon cancer. Therefore, we performed a correlated mRNA expression analysis of COX2 and 5-LOX as well as of VEGF-A, VEGF-B, VEGF-C and PlGF in colon carcinoma samples and colon adenoma samples. VEGF-D was not included in this study because previous results revealed invariable expression levels of *VEGF-D* in colon cancer [10].

### 6.1.2. Materials and methods

**Biological samples**

Samples were obtained from colon adenoma (n=16) and primary colon carcinoma (n= 37 ) at the Ghent University Hospital. Adenoma and carcinoma were sampled by biopsy in the infiltrating area of the growth. Histopathological examination confirmed the adenoma/carcinoma state of the

tissue. From each patient, a corresponding healthy colon sample was taken from the same colon segment. None of the patients had received chemo- or radiotherapy before colonoscopy. Tissue samples were collected immediately after isolation in RNA*later*® Solution (Ambion/Applied Biosystems, Foster City, CA). All samples were kept at -80°C until RNA extraction. The clinicopathological features of the patients are summarized in tables 6.1 and 6.2. All tissues were obtained following informed consent of the patients and approval of the study by the Ethics Committee of the Ghent University Hospital.

| **Variable** | **Number of patients** |
|---|---|
| Sex | |
| Male | 21 |
| Female | 16 |
| Age at diagnosis | |
| Median age (range, years) | 71 (39–85) |
| Site of tumor | |
| Sigmoid | 20 |
| Colon descendens | 1 |
| Colon transversum | 2 |
| Hepatic flexure | 1 |
| Colon ascendens | 5 |
| Caecum | 7 |
| Not specified | 1 |
| Differentiation grade | |
| Low | 12 |
| Moderate | 17 |
| High | 3 |
| Unknown | 5 |
| Dukes classification | |
| Dukes' A | 3 |
| Dukes' B | 15 |
| Dukes' C | 7 |
| Dukes' D | 12 |
| Unknown | 0 |
| T category | |
| T1-T2 | 3 |
| T3-T4 | 26 |
| Tx | 8 |
| Lymphatic spread | |
| N0 | 18 |
| N+ | 10 |
| Nx | 9 |
| Metastasis | |
| M0/Mx | 25 |
| M+ | 12 |

**Table 6.1 - Clinicopathological features of the colon carcinoma patients.**

| Variable | Number of patients |
|---|---|
| Sex | |
| Male | 10 |
| Female | 6 |
| Age at diagnosis | |
| Median age (range, years) | 68 (18-80) |
| Site of tumor | |
| Sigmoid | 9 |
| Colon transversum | 2 |
| Hepatic flexure | 1 |
| Colon ascendens | 1 |
| Caecum | 3 |
| Dysplasia | |
| Low | 10 |
| High | 4 |
| Unknown | 2 |
| Classification | |
| Tubular | 8 |
| Tubulovillous | 6 |
| Villous | 1 |
| Unknown | 1 |

**Table 6.2 - Clinicopathological features of the colon adenoma patients.**

### RNA-extraction, RNA-quality control and cDNA-synthesis

Total RNA was extracted with the RNeasy Plus mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. This kit contains a gDNA-elimination step to avoid gDNA contamination. After extraction, RNA quality and integrity were verified using an RNA 6000 Nano Chip Kit on the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Only samples with adequate quality and integrity (adenoma 16/18, carcinoma 37/39) were used for the RT-qPCR analysis. cDNA was synthesized from 1 µg of total RNA using Superscript® II reverse transcriptase (Invitrogen, Merelbeke, Belgium) according to the manufacturer's instructions.

### RT-qPCR

Real-time quantitative PCR (RT-qPCR) was performed using the LC 480 Sybr Green I master kit on a LightCycler® 480 Real-Time PCR system (both from Roche Applied Science, Penzberg, Germany). Primers were designed using PrimerSelect (DNASTAR, Madison, USA) and purchased from Invitrogen. The primers were designed for gene-specific expression profiling and cover all splice variants. The sequences of the forward and reverse primers were as follows: VEGF-A 5'-TGAGTTGCCCAGGAGACCAC-3' and 5'-GAAGGGGAGCAGGAAGAGGAT-3'; VEGF-B 5'-CCGGAAGCTGCGAAGGTGACA-3' and 5'-

GGGAGACAAGGGATGGCAGAAGAG-3'; VEGF-C 5'-CACGGCTTATGCAAGCAAAGA-3' and 5'-TCCTTTCCTTAGCTGACACTTGT-3'; PlGF 5'-TGCGGCGATGAGAATCTGC-3' and 5'-AGCGAACGTGCTGAGAGAAC-3'; COX2 5'-TTGCTGGAACATGGAATTACC-3' and 5'-TGCCTGCTCTGGTCAATG-3'; 5-LOX 5'-TGGCGCGGTGGATTCATAC-3' and 5'-CAGGGGAACTCGATGTAGTCC-3'. All samples were assayed in triplicate. Relative expression values were calculated using the 2(-delta delta C(T)) method and were normalized against reference genes: tata-binding protein (*TBP*) and succinate dehydrogenase complex subunit A (*SDHA*) (primers: TBP 5'-CGGCTGTTTAACTTCGCTTC-3' and 5'-CACACGCCAAGAAACAGTGA-3'; SDHA 5'-TGGGAACAAGAGGGCATCTG-3' and 5'-CCACCACTGCATCAAATTCATG-3'). In these calculations we took into account the PCR efficiency of the individual PCR reactions, calculated on the basis of linear regression as described in Ruijter et al [18]. The normalized relative expression values were scaled against the median of the respective healthy tissue samples (median of healthy samples set to 1). The specificity of amplification was confirmed by evaluation of the melting curves.

**Statistical analysis**

Statistical analysis was performed using GraphPad Prism® software (GraphPad Software Inc., La Jolla, California, USA). For comparisons of paired samples, the Wilcoxon signed rank test was used. Statistical significance of comparisons between two independent groups was determined with the two-tailed Mann-Whitney U test. Correlations between the expression levels of genes were calculated with the Spearman's correlation test. Significant p-values were ranked as $p<0.05$ (*), $p<0.01$ (**) and $p<0.001$ (***).

### 6.1.3 Results

**Stage-dependent expression profiles of eicosanoid enzymes**

n-Fold induction values of *COX2* and *5-LOX* were determined for colon adenoma and carcinoma samples with respect to matched healthy tissue samples. *COX2* was significantly induced only in carcinoma samples (Fig 6.1A). *5-LOX* on the other hand was significantly increased in both adenoma and carcinoma stages albeit at a significantly higher level in adenoma samples compared to carcinoma samples (Fig 6.1B).

In a next step, we compared the *COX2* and *5-LOX* expression signatures from individual colon adenoma and carcinoma samples with their matched healthy samples. The cut-off for

overexpression was set on the median +(2.58 x SD) of the corresponding healthy tissue cohort. *5-LOX* was overexpressed in 75% of the adenoma samples either alone (37,5%) or in combination with *COX2* (37,5%) (Figure 6.1D). *COX2* on the other hand was only overexpressed in addition to *5-LOX* (Figure 6.1C). Strikingly, carcinoma samples revealed opposite expression profiles with overexpression of *COX2* in almost every sample either alone (48,5%) or in combination with *5-LOX* (43%), whereas *5-LOX* was only overexpressed in samples with *COX2* overexpression (Figure 6.1).

Combined, these results indicate that adenoma samples have a predominantly 5-LOX profile while carcinoma samples mainly have a COX2 mRNA expression profile.

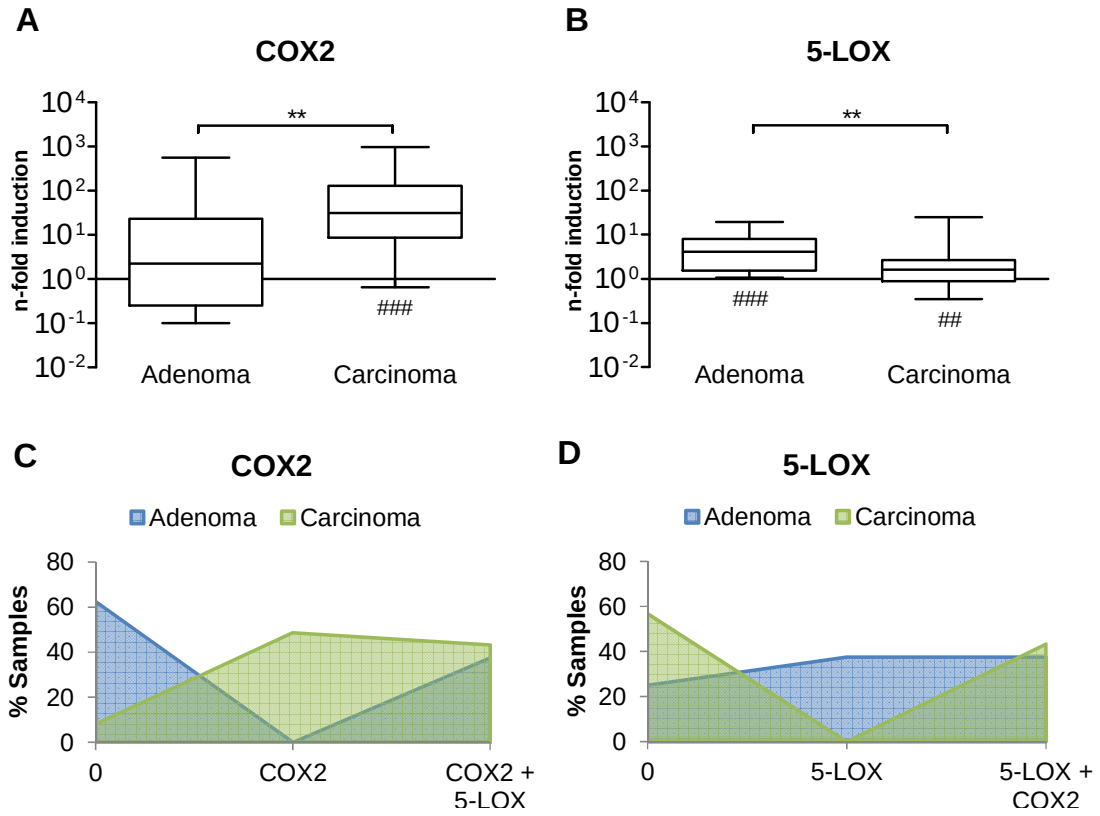



**Figure 6.1 – The induction of COX2 and 5-LOX during progression of colon cancer.** n-Fold induction levels in adenoma and carcinoma samples of **(A)** COX2 and **(B)** 5-LOX are shown in the graphs above. The n-fold induction value represents the ratio of the expression value of the diseased sample against the expression value of the paired healthy sample. The box represents the median with interquartile range and the whiskers represent minimum and maximum ratios. Significance of the induction on its own is indicated beneath the minimum whisker with ##: $p<0.01$ and ###: $p<0.001$, calculated with Wilcoxon signed rank test. Significant differences in induction between the stages is indicated above the boxes with **: $p<0.01$; ***: $p<0.001$, calculated with Mann-Whitney U test.
The graphs below show for COX2 **(C)** and 5-LOX **(D)** the percentages of colon adenoma and carcinoma samples which are either negative or overexpressing one or both eicosanoid enzymes. The cut-off for overexpression was determined based on the median of the healthy samples for each population group +(2.58 x SD).

### Dual expression of COX2 and 5-LOX is associated with increased risk of malignant transformation in adenoma

Colon adenomas showing a high grade of dysplasia and featuring a villous morphology have an increased risk of further progressing to the carcinoma stage. In the current study, 16% of adenoma samples had high grade dysplastic or villous features. Strikingly, as shown in figure 6.2 all of these samples displayed a dual *COX2* and *5-LOX* profile. Moreover, high-risk-adenoma samples represented 66% of the adenoma samples overexpressing both *COX2* and *5-LOX*. Thus, a dual COX2 and 5-LOX eicosanoid enzyme profile is strongly associated with an increased risk of malignant transformation into carcinoma.

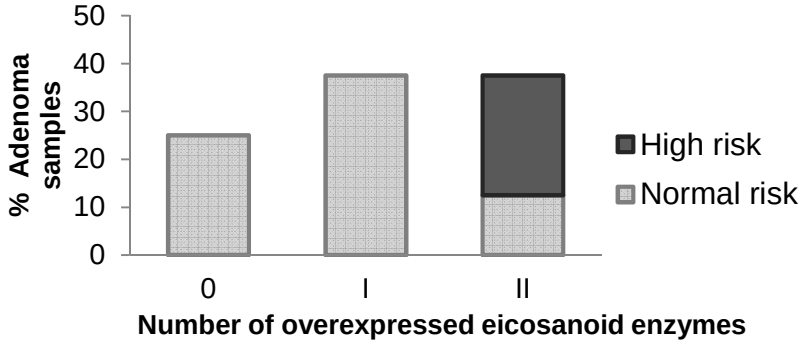
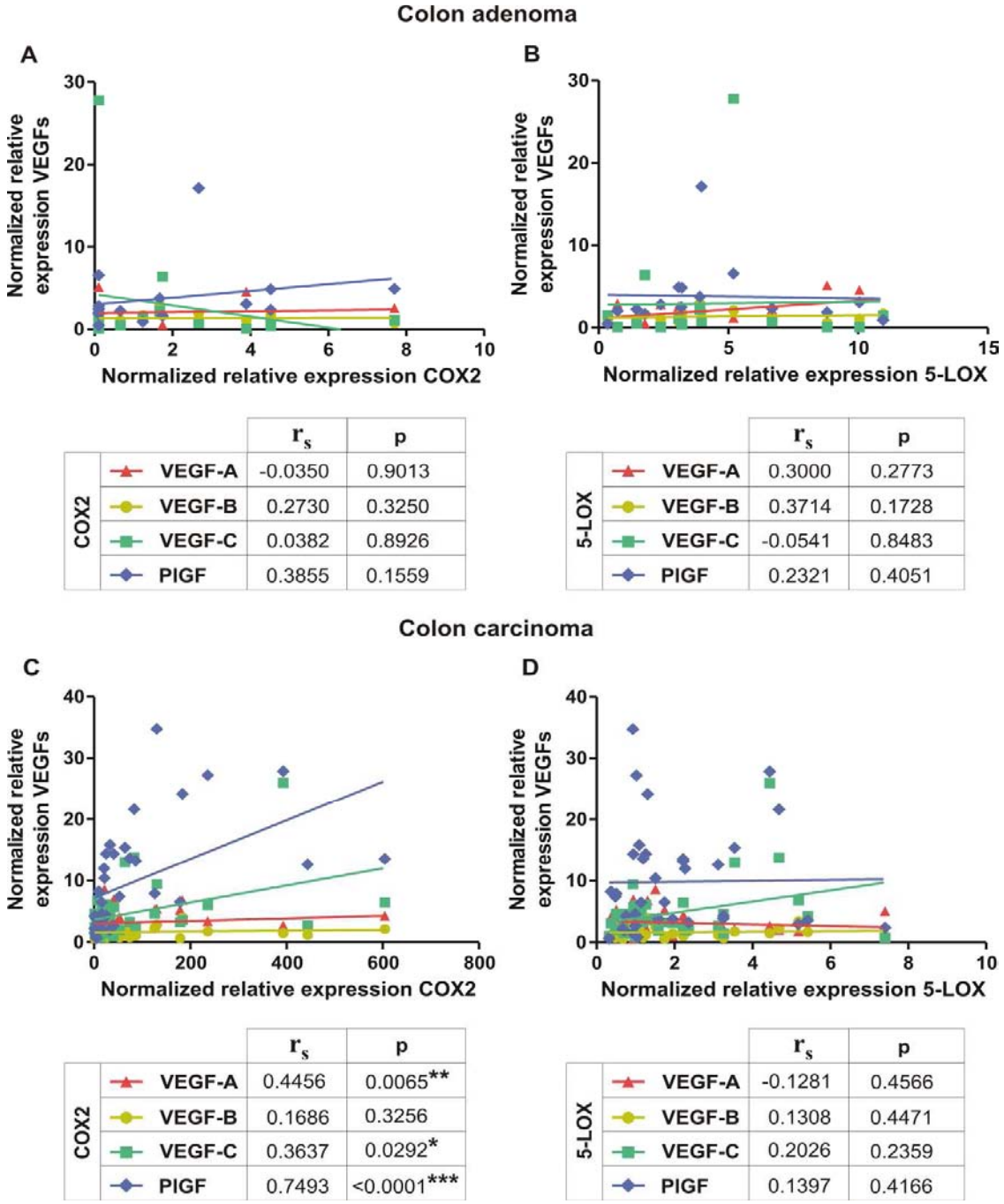



**Figure 6.2 - Risk of malignant transformation versus number of overexpressed eicosanoid enzymes in colon adenoma samples.** The cut-off for overexpression was determined based on the median of the healthy samples for each population group +(2.58 x SD).

### Correlation with angiogenic gene expression

Especially COX2-produced prostanoids but also leukotrienes produced by 5-LOX have been shown to promote angiogenesis through the induced expression of especially VEGF-A. We showed the occurrence of *PlGF* and *VEGF-A* overexpression already at the adenoma stage with an additional overexpression of *VEGF-B* and *VEGF-C* in the carcinoma stage. To verify whether the overexpression of *COX2* and *5-LOX* is correlated with the overexpression of VEGF family members, we performed a Spearman's correlation analysis. As shown in figure 6.3, colon carcinoma samples showed significant correlations between the overexpression of *COX2* and the overexpression of *VEGF-A* and *PlGF*, and to a lesser extent of *VEGF-C*. In contrast, in adenoma samples no significant correlations between *COX2* overexpression and overexpression of *VEGFs* emerged. Also for *5-LOX*, no significant correlations appeared in either of the sample cohorts. This analysis indicates that an inflammatory *COX2*-associated expression of *VEGFs* may exist in colon carcinoma, but not in colon adenoma.




| | | $r_s$ | p |
|---|---|---|---|
| COX2 | VEGF-A | -0.0350 | 0.9013 |
| | VEGF-B | 0.2730 | 0.3250 |
| | VEGF-C | 0.0382 | 0.8926 |
| | PIGF | 0.3855 | 0.1559 |

| | | $r_s$ | p |
|---|---|---|---|
| 5-LOX | VEGF-A | 0.3000 | 0.2773 |
| | VEGF-B | 0.3714 | 0.1728 |
| | VEGF-C | -0.0541 | 0.8483 |
| | PIGF | 0.2321 | 0.4051 |

| | | $r_s$ | p |
|---|---|---|---|
| COX2 | VEGF-A | 0.4456 | 0.0065** |
| | VEGF-B | 0.1686 | 0.3256 |
| | VEGF-C | 0.3637 | 0.0292* |
| | PIGF | 0.7493 | <0.0001*** |

| | | $r_s$ | p |
|---|---|---|---|
| 5-LOX | VEGF-A | -0.1281 | 0.4566 |
| | VEGF-B | 0.1308 | 0.4471 |
| | VEGF-C | 0.2026 | 0.2359 |
| | PIGF | 0.1397 | 0.4166 |

**Figure 6.3 - Correlation between overexpression of VEGF family members and overexpression of eicosanoid enzymes in colon adenoma and carcinoma samples.** Relative mRNA expression levels of VEGF-A (▲), VEGF-B (●) , VEGF-C (■) and PlGF (◆) versus those of **(A, C)** COX2 and **(B, D)** 5-LOX are shown in scatter plots for **(A-B)** colon adenoma and **(C-D)** colon carcinoma. The best-fit line is depicted for each gene pair. Expression levels were normalized against reference gene TBP and SDHA and were scaled against the median of the paired healthy colon samples (median set to 1). The tables underneath the scatter plots summarize the results of the Spearman's correlation analysis with $r_s$ representing the Spearman correlation coefficient which ranges from 1 to -1 with 1 standing for a perfect correlation, 0 for no correlation, and -1 for a perfect inverse correlation. The p-value (p) quantifies the likelihood that the correlation is found by chance and the variables don't really correlate. Significant correlations are indicated by *: $p<0.05$, **: $p<0.01$ and ***: $p<0.001$.

**Co-expression of VEGF family members coincides with overexpression of eicosanoid enzymes**

*VEGF* expression signatures from individual colon adenoma and carcinoma samples were determined, applying a similar cut-off for overexpression or not for *COX2* and *5-LOX* above. Combining both sets of expression signatures then allowed us to picture to what extent the shift toward co-expression of *VEGFs* observed upon progression from colon adenoma to colon carcinoma coincides with the overexpression of eicosanoid enzymes. Strikingly, this combined analysis revealed a strong interdependency between overexpression of VEGF family members and overexpression of *COX2* and/or *5-LOX*. Co-expression of VEGFs occurs almost exclusively in colon adenoma and carcinoma samples which have also eicosanoid enzymes overexpressed (Figure 6.4A, C). However, neither adenoma nor carcinoma samples achieved statistical significance in their contingency tables, though the p-values were low, 0.052 and 0.086 respectively (Table 6.3). Nevertheless, when regarding the proportional presence of eicosanoid enzyme overexpression in the group of samples overexpressing none or one VEGF and in the group with co-expression (Figure 6.4B, D), it clearly shows that co-expression of VEGF family members associates with the presence of overexpressed eicosanoid enzymes.

| **Adenoma** | | **Eicosanoid enzymes** | | |
|---|---|---|---|---|
| | | **Absent** | **Present** | Total |
| **VEGFs** | **No or 1** | 4 | 6 | 10 |
| | **Co-expression** | 0 | 6 | 6 |
| | Total | 4 | 12 | 16 |

| **Carcinoma** | | **Eicosanoid enzymes** | | |
|---|---|---|---|---|
| | | **Absent** | **Present** | Total |
| **VEGFs** | **No or 1** | 2 | 5 | 7 |
| | **Co-expression** | 1 | 29 | 30 |
| | Total | 3 | 34 | 37 |

**Table 6.3 – Contingency tables for VEGF family members and eicosanoid enzymes** for colon adenoma and carcinoma samples. Statistical significance was calculated with Fisher's exact test.

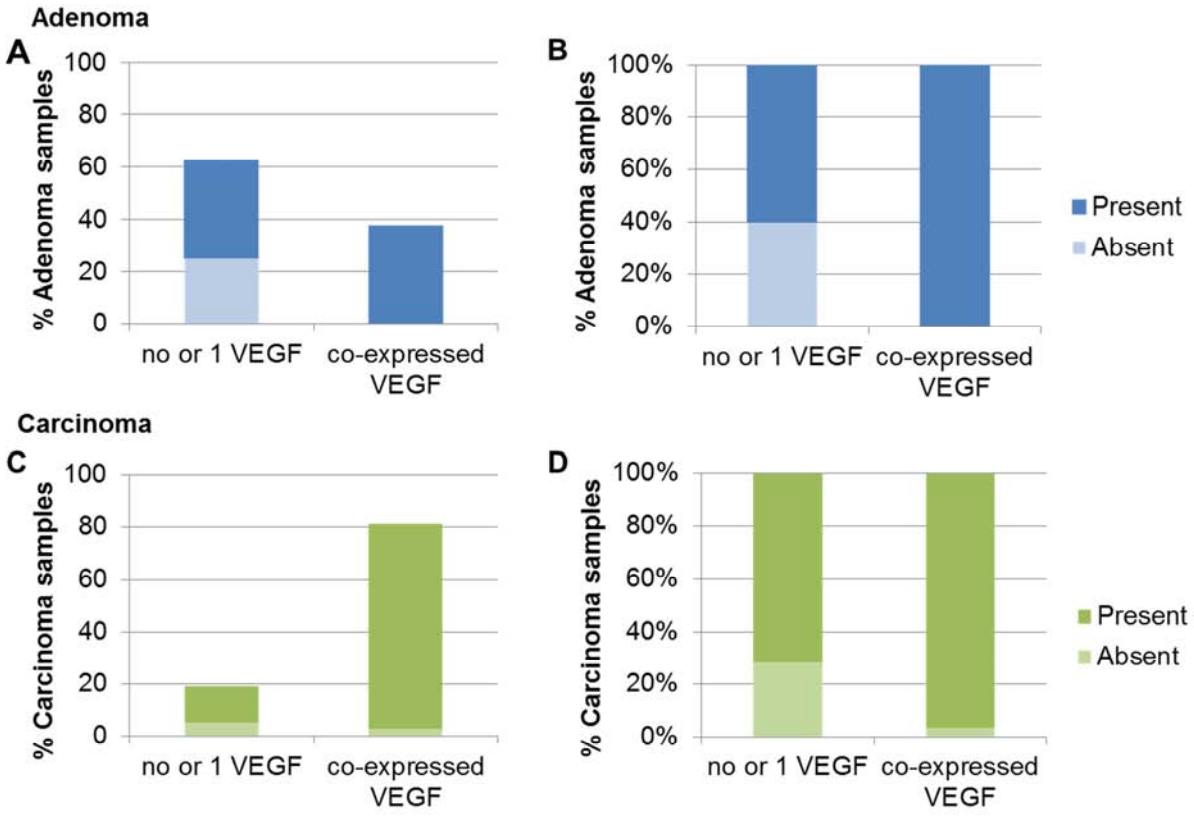



**Figure 6.4 - Correlation between co-expression of VEGFs and overexpression of eicosanoid enzymes for individual colon adenoma and carcinoma patients.** The graphs show the percentages of colon adenoma **(A)** and carcinoma **(C)** samples that overexpressed none or one VEGF or multiple VEGF family members, concomitant with the absence or presence of overexpressed eicosanoid enzymes. Also the proportional presence of overexpressed eicosanoid enzymes within each VEGF-signature-group is shown for colon adenoma (**B**) and carcinoma (**D**) samples. 'Absent" means no eicosanoid enzymes were overexpressed, "present" means that either COX2, 5-LOX or both were overexpressed. "Co-expressed" means that 2, 3 or 4 VEGF members were overexpressed. The cut-off for overexpression was determined based on the median of the healthy samples for each cohort +(2.58 x SD).

### 6.1.4 Discussion

Previous studies on the correlation of the expression of eicosanoid enzymes and VEGF family members in (colon) cancer are strongly focused on COX2 and VEGF-A along with VEGF-C [3, 11-17]. Yet, it remains unclear to what extent overexpression of VEGF-B and PlGF correlate with COX2 overexpression and if any correlation can be found with 5-LOX. Here, we performed a correlated mRNA expression analysis of COX2, 5-LOX and VEGF family members during the progression from colon adenoma towards carcinoma.

Already at the early stage of colon adenoma, a distinctive inflammatory profile with a strong expression increment of 5-LOX was apparent. Overexpression of 5-LOX has been proposed

before to be present in colon adenomas [19, 20]. Similarly an induction of COX2 in adenoma samples has been described [21-23]. Also we detected *COX2* overexpression but only in combination with *5-LOX* and mainly restricted to adenoma samples showing a villous morphology and/or exhibiting a high degree of dysplasia, two characteristics indicative of greater risk of malignant transformation. Applying the dual expression of *COX2* and *5-LOX* as a marker for malignancy, we thus could identify all adenoma samples at higher risk of malignant transformation. Conversely, two thirds of the samples with dual expression were marked by an independent pathological examination as being at higher risk. Yet, since also *COX2* expression per se correlated with the grade of dysplasia (supplemental Table S6.1), it is not possible to state at this point whether the observed association with increased risk is caused by the dual *COX2* and *5-LOX* overexpression or by *COX2* alone. Expansion of this analysis to larger cohort size may be indicated to fully resolve this issue of obvious importance for chemoprevention of colon cancer. The finding by Mohammed and colleagues that treatment of $Apc^{-/+}$ mice with the dual 5-LOX/COX2 inhibitor, licofelone, prevented successfully the development of colon adenomas and outgrowth of colon carcinomas [24] clearly corroborates our proposition of an association between high-risk adenoma phenotypes and the occurrence of a dual *COX2* and *5-LOX* overexpression.

Also colon carcinoma samples showed an inflammatory signature on the basis of a pronounced *COX2* expression increment in almost all samples. However, the *5-LOX* increment observed in adenoma samples was less apparent. Looking for inflammatory pro-angiogenic triggers contributing to the observed expression profiles of VEGF family members [10], we found a strong correlation between COX2 and VEGF-A mRNA levels in colon carcinoma samples by means of Spearman's correlation analysis, thus confirming previous reports on the association of COX2 expression with expression of VEGF-A [11, 25]. Interestingly, this analysis also revealed a novel and strong association between COX2 and PlGF. However, although also reported in literature, we observed only a weak, yet statistically significant association between COX2 and VEGF-C [3, 12]. Finally, from adenoma samples no association between VEGF genes and eicosanoid enzyme genes emerged. Apparently, a non-inflammatory/non-eicosanoid mechanism underlies the expression of single VEGF family members in colon adenomas.

These observed correlations in carcinoma samples between overexpressed *COX2* and increased mRNA levels of especially VEGF-A and PlGF may indicate that eicosanoids are implicated in

the cumulative co-expression of multiple VEGF family members during progression from colon adenoma toward colon carcinoma. Mapping the *VEGF* signatures against the presence of eicosanoid enzymes indeed revealed in colon carcinoma samples a clear convergence of the overexpression of multiple VEGF family members with the presence of overexpressed eicosanoid enzymes. Strikingly, such convergence was also observed in colon adenoma samples overexpressing two or more *VEGFs*. As a whole, this correlation analysis strongly points to a role for COX2- and/or 5-LOX-derived eicosanoids in driving the aberrant expression of multiple VEGF family members in both adenoma and carcinoma stages of colon cancer but not or to a lesser extent the physiological expression of single VEGF family members.

In conclusion, this correlated analysis of *COX2, 5-LOX* and *VEGFs* mRNA expression levels reveals the complex and intertwined nature of inflammatory and angiogenic gene expression already at the stage of adenoma and upon further progression to carcinoma. Thus, our expression analysis identifies *5-LOX* as the predominant eicosanoid enzyme overexpressed in colon adenoma and *COX2* in colon carcinoma. In both stages, the occurrence of overexpressed eicosanoid enzymes is associated with the cumulative co-expression of multiple *VEGFs.* In addition, dual expression of *COX2* and *5-LOX* in colon adenoma is correlated with an increased risk of malignant transformation, thus identifying both enzymes as clinically relevant targets for chemoprevention as well as treatment of colon cancer.

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## SUPPLEMENTAL DATA

| | COX2 | 5-LOX | VEGF-A | VEGF-B | VEGF-C | PlGF |
|---|---|---|---|---|---|---|
| **Age**[1] | 0.5040 | 0.8749 | 0.7925 | 1.000 | 0.3530 | 0.1806 |
| **Gender**[2] | 0.1063 | 0.2198 | 0.7925 | 0.2198 | 0.9130 | 0.3676 |
| **Localization**[3] | 0.6407 | 0.1852 | 0.2418 | 0.1846 | 0.2912 | 0.4128 |
| **Dysplasia**[4] | 0.0267* | 0.6354 | 0.3736 | 0.3037 | 0.2273 | 0.6354 |
| **Histology**[5] | 0.7683 | 0.7789 | 0.0401* | 0.0205* | 0.0228* | 0.3969 |

[1] Comparison of expression levels in patients younger than 70 years versus patients (n=10) of 70 years or older (n=6) with Mann-Whitney test.

[2] Comparison of expression levels in male (n=10) versus female (n=6) patients with Mann-Whitney test.

[3] Comparison of expression levels in samples from different adenoma sites (caecum and Valve of Bauhin (n=3) versus colon ascendens, transversum, descendens and hepatic flexure (n=4) versus sigmoid (n=9)) with Kruskal Wallis test.

[4] Comparison of expression levels in samples with low grade (n=10) versus high grade (n=4) dysplasia with Mann-Whitney test. *: p<0.05

[5] Comparison of expression levels in samples from tubular (n:-=8) versus villous/tubulovillous (n=7) adenoma with Mann-Whitney test. *: p<0.05

**Additional table S6.1 - Comparison of the expression of COX2, 5-LOX and VEGF family members with clinicopathological features of colon adenoma.**

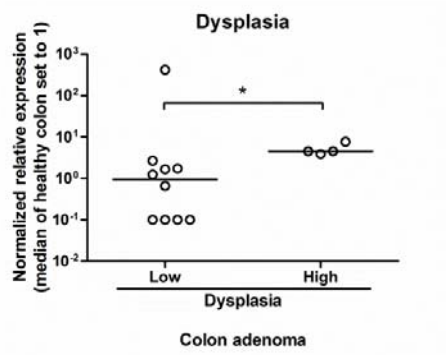


**Figure S6.1 – Comparison of COX2 expression levels in colon adenoma with low versus high dysplasia** with Mann-Whitney test. *:p<0.05

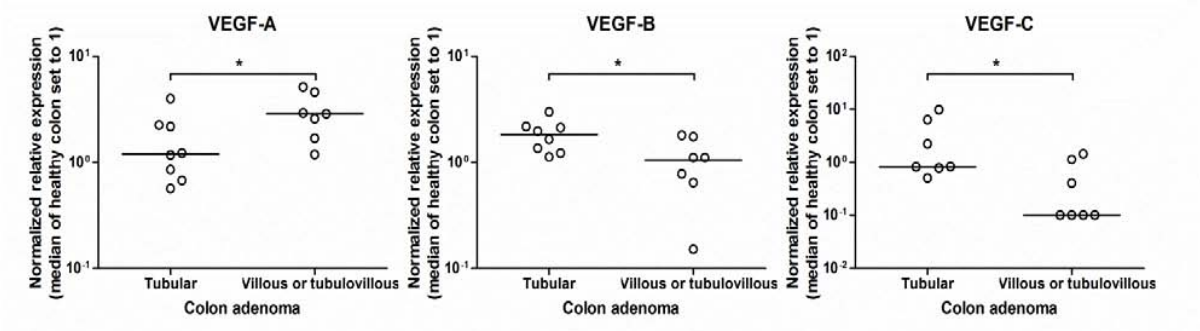


**Figure S6.2 - Comparison of expression levels in tubular adenoma versus villous or tubulovillous adenoma** for VEGF-A, VEGF-B and VEGF-C with Mann-Whitney test. *: p<0.05

## 6.2 Additional data

### 6.2.1 Eicosanoids promote invasion of human colon carcinoma cells

In order to elucidate the mechanism behind the observed mRNA signatures of COX2, 5-LOX and VEGF-family members in human clinical samples (section 6.1), we aimed to re-translate these observations to more basic *ex vivo* or *in vitro* scientific models. A first approach hereto was to determine the effect of eicosanoids on malignancy of human colon carcinoma cell lines. This suggestion is based on the observed correlation between the dual overexpression of *COX2* and *5-LOX* with an increased risk of malignant transformation in adenoma. Because malignancy is not a measurable *in vitro* parameter, invasiveness was used as a criterion for malignancy. After all, the capability to invade is a critical feature that distinguishes malignant cells from benign cells. Eicosanoid agonists, $PGE_2$ and $LTD_4$, as well as COX2 and 5-LOX inhibitors, meloxicam and zileuton, were tested for their effect on invasiveness of colon carcinoma cells using an *in vitro* invasion assay. The invasion assay was based on the preparation of a native collagen type I gel on top of which colon carcinoma cells were seeded as single cells [1]. After culture for 24 hours, the single-cell invasion was evaluated with an inverted phase-contrast microscope and quantified by calculating the invasion index (the number of cells with invasive extensions/the total number of cells counted x 100) for a total of 10 fields.

The experimental setup consisted of the human colon carcinoma cell lines HCT-8 (HCT-8/E11), HCT-116, DLD-1 and HT29, stimulated with $PGE_2$ and/or $LTD_4$ or inhibited with meloxicam and/or zileuton. In order to determine the effect of either $PGE_2$ or $LTD_4$ alone, $PGE_2$ was added to the cells together with zileuton to block the endogenous production of leukotrienes, and $LTD_4$ was added with meloxicam to block the endogenous production of prostaglandins. As positive control, the cells were treated with TGF-α.

The obtained results indicated that HCT-116 and HT-29 are not suitable for this invasion assay. HCT-116 cells showed massive invasion in every condition, including the negative control (Figure 6.5C,D). In contrast, no invasion was observed with HT-29 cells in any of the conditions assayed (data not shown). DLD-1 and HCT-8 cells showed however a clear induction of invasion after treatment with TGF-α (Figure 6.5A,B). In DLD-1 cultures, a substantial amount of invasive

cells was already present in the negative control sample, leading to a smaller window of measurement.

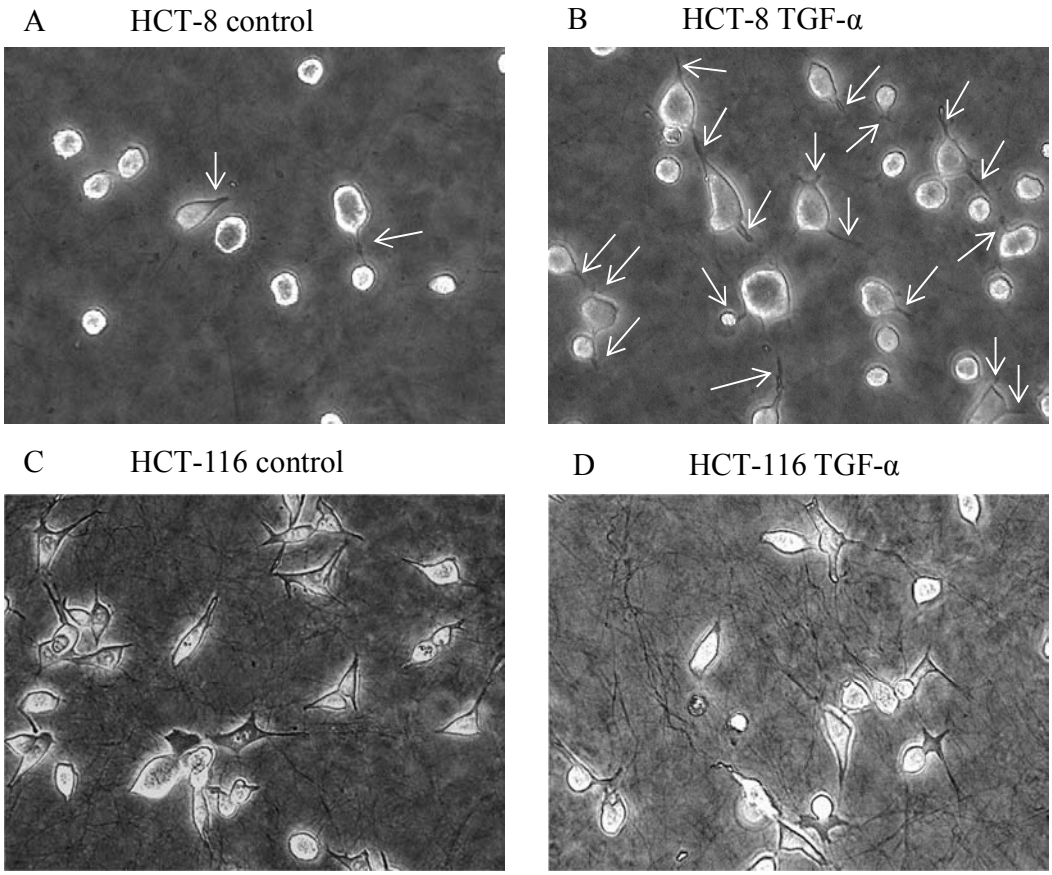


**Figure 6.5 – Representative pictures of HCT-8 and HCT-116 cell invasion.** Phase contrast pictures of single HCT-8 (A, B) and HCT-116 (C, D) colon cancer cells seeded on collagen type I gel and cultured for 24 h in culture medium (negative control) or culture medium supplemented with TGF-α (10ng/ml) (positive control). Arrows indicate invasive extensions in A and B.

As shown in figure 6.6, both $PGE_2$ and $LTD_4$ promoted invasion of the HCT-8 and DLD-1 cells to a similar extent as the TGF-α positive control. Only a limited additional invasion was observed when both eicosanoids were added to the cultures. In contrast, both zileuton and meloxicam had minimal effects on the invasion of both cell types. Interestingly, inhibition of COX2 abolished the induction of invasion by $LTD_4$ and conversely, the promotion of invasion by $PGE_2$ was abolished by zileuton.

The promotion of invasion by $PGE_2$ agrees well with previous studies reporting a $PGE_2$-promoted invasion of colon cancer cells [2, 3]. No published data were found for $LTD_4$. Strikingly, the inhibition of the alternate eicosanoid pathway, abolished the increased invasion by

both $PGE_2$ and $LTD_4$ although the addition of both eicosanoids did only barely induce an additional invasion, thus indicating interdependence rather than synergy between COX2- and 5-LOX-pathways for the stimulation of invasion.

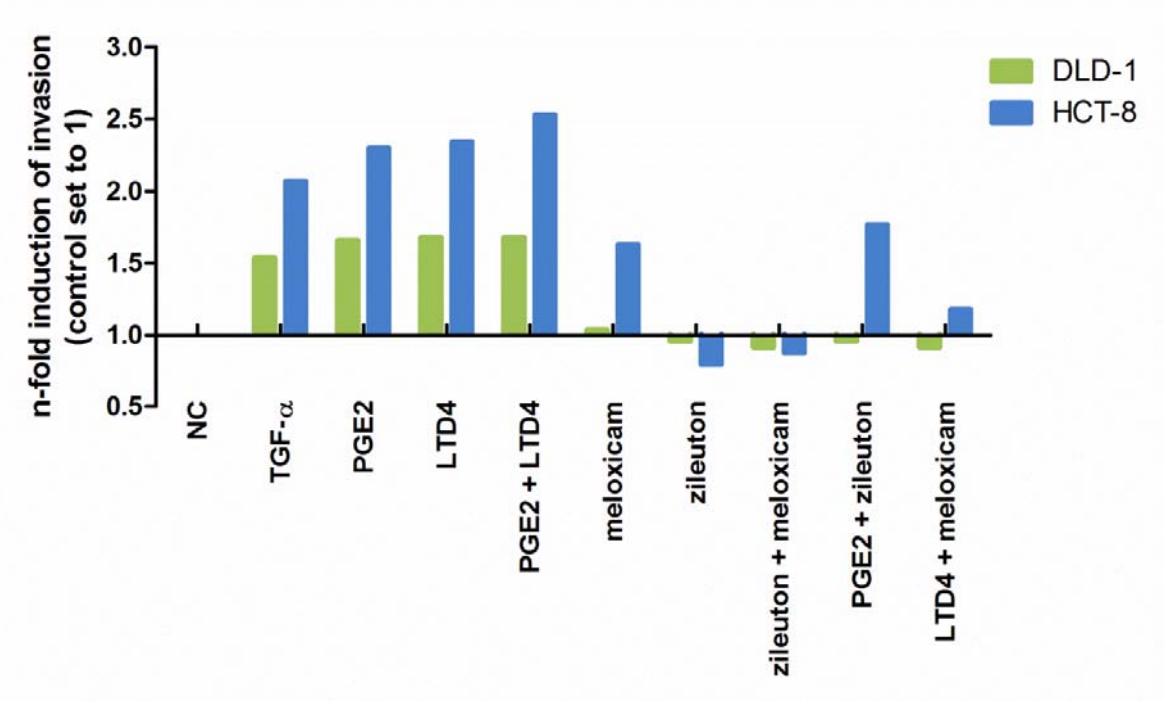


**Figure 6.6 – $LTD_4$ and $PGE_2$ promote invasion in colon cancer cells.** The n-fold induction of invasion is shown for HCT-8 and DLD-1, with reference to the invasion index of the negative control (NC), which was set to 1. Used concentrations were: TGF-α [1 µM], $PGE_2$ [1 µM], $LTD_4$ [1 µM], meloxicam [10µM] en zileuton [10µM].

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In conclusion, these results show for the first time that next to $PGE_2$, also $LTD_4$ may promote invasiveness of colon cancer cells. However, the observed correlation in adenoma samples between increased risk of malignant transformation and dual overexpression of COX2 and 5-LOX, was only partially found because dual administration of $PGE_2$ and $LTD_4$ did not show an additional effect. Yet, suppression of either factor inhibited the invasion promoting effect of the other, indicating interdependence but not synergy between both eicosanoid pathways for the promotion of colon cancer cell invasiveness.

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### 6.2.2 Eicosanoids don't affect *COX2, 5-LOX* and *VEGFs* expression patterns in human colon carcinoma cells

In a second approach to re-translate our observations on clinical samples to more basic *ex vivo* or *in vitro* scientific models, we verified to what extent COX2- and 5-LOX-derived eicosanoids may impact *VEGF* expression levels. Based on the results presented in section 6.1, we hypothesized that the administration of eicosanoids ($PGE_2$ or $LTD_4$) may induce the co-expression of *VEGFs*, whereas the administration of COX2- and 5-LOX-inhibitors (meloxicam or zileuton) may inhibit their expression. The human colon carcinoma cell lines HT-29, HCT-116 and CaCo-2 were selected for this experiment because of their distinct behavior. Whereas CaCo-2 is a slow growing cell line, HCT-116 behaves rather aggressive in the invasion assay described above (see 6.2.1). HT-29 is a less aggressive, though still a rapidly proliferating cell type. Cells were seeded at 1.25 $x10^5$ cells in twelve-well plates and cultured for 24 hours before administration of eicosanoid agonists or antagonists. TNF-α was added as a positive control and DMSO was added as mock control. After one, three and six hours of treatment, RNA was isolated and cDNA-synthesis followed by RT-qPCR were performed as described before (see 6.1.2).

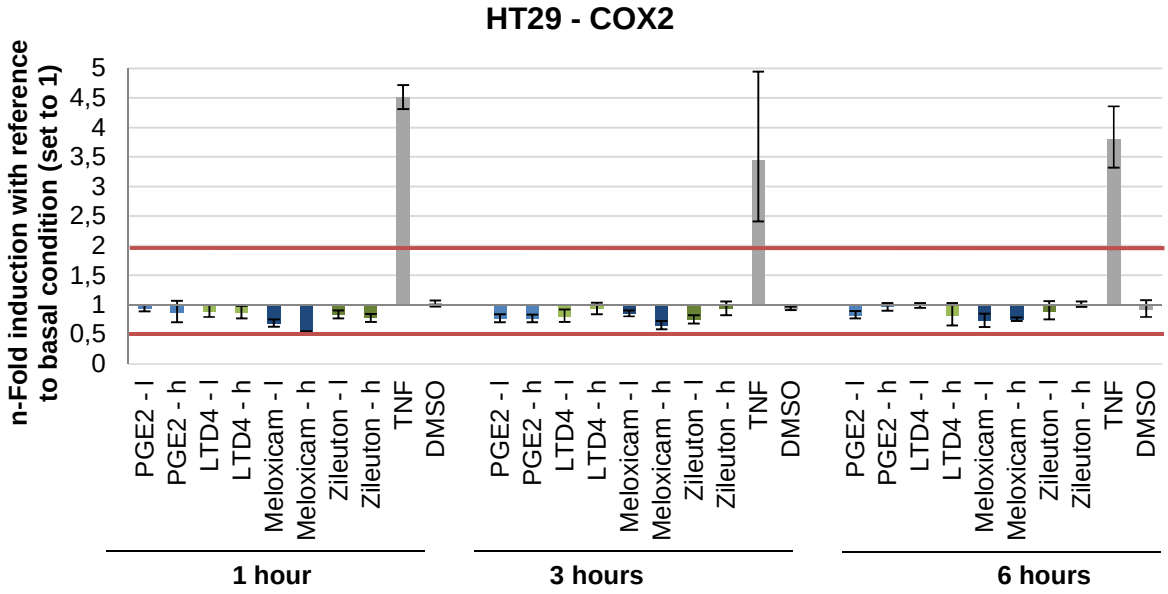


**Figure 6.7 – COX2 expression is induced by TNF in HT-29 colon carcinoma cells.** n-Fold induction levels of *COX2* in the HT-29 human colon carcinoma cell line is shown. The n-fold induction value represents the ratio of the expression value of the treated sample against the expression value of the basal condition. 0.5 and 2-fold inductions (red lines) were evaluated as respectively significant reduction and induction. Treatment conditions were: $PGE_2$-l (low): 0.5 µg/ml, $PGE_2$-h (high): 5 µg/ml, $LTD_4$-l: 0.05 µg/ml, $LTD_4$-h: 0.5 µg/ml, meloxicam-l: 5 µg/ml, meloxicam-h: 10 µg/ml, zileuton-l: 5 µg/ml, zileuton-h: 10 µg/ml, TNF-α: 250 IU/ml (positive control), DMSO, 2µl/ml (mock control).

As expected, a significant induction of *COX2* was observed for the treatment condition with TNF-α at all three time points in cell line HT-29 (Figure 6.7). However, the other cell lines, DLD-1 and Caco-2, showed no induction of *COX2* after treatment with TNF-α (data not shown). In addition, none of the other treatment conditions had an effect on the expression of *COX2* in the three cell lines. To our surprise, also the expression levels of *5-LOX*, *VEGF-A*, *VEGF-B* or *PlGF* were not affected by any treatment condition in all three cell lines. Figure 6.8 shows the expression of *PlGF* in CaCo-2 as a representative example. Similar graphs were obtained for all the genes – VEGF-A, VEGF-B, PlGF, COX2 and 5-LOX – in the three cell lines assayed. In addition, *VEGF-C* expression was below the detection limit in all three cell lines.

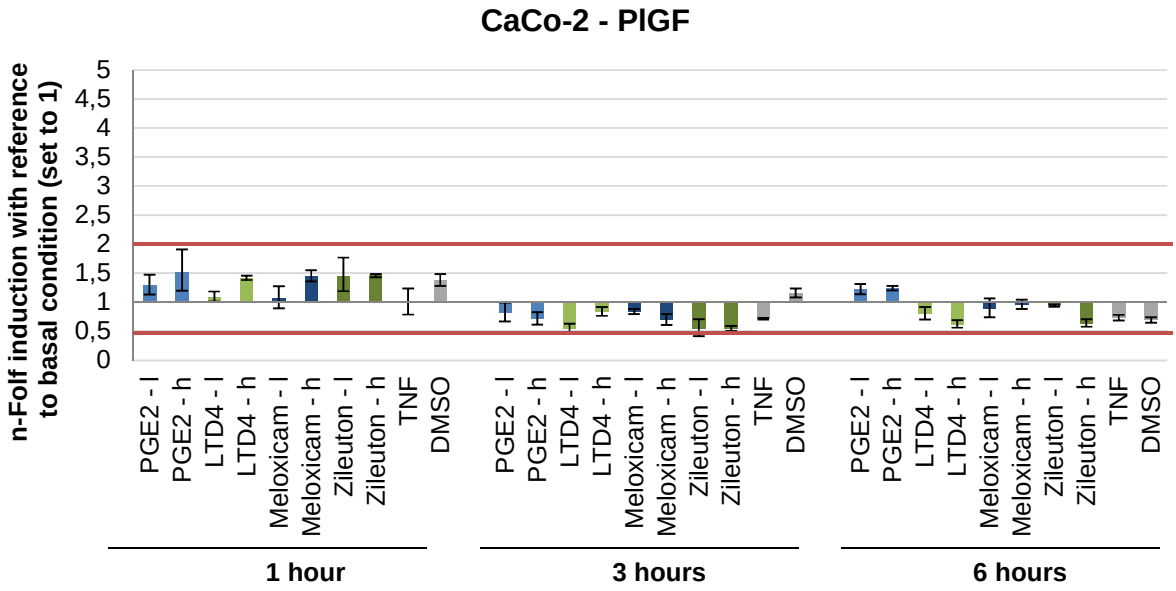


**Figure 6.8 – PlGF mRNA expression levels in CaCo-2 cells.** n-Fold induction levels of *PlGF* in the CaCo-2 human colon carcinoma cell line is shown. The n-fold induction value represents the ratio of the expression value of the treated sample against the expression value of the basal condition. 0.5 and 2-fold inductions (red lines) were evaluated as respectively significant reduction and induction. Treatment conditions were: $PGE_2$-l (low): 0.5 µg/ml, $PGE_2$-h (high): 5 µg/ml, $LTD_4$-l: 0.05 µg/ml, $LTD_4$-h: 0.5 µg/ml, meloxicam-l: 5 µg/ml, meloxicam-h: 10 µg/ml, zileuton-l: 5 µg/ml, zileuton-h: 10 µg/ml, TNF-α: 250 IU/ml (positive control), DMSO, 2µl/ml (mock control).

These (negative) results clearly show that the expression signatures observed in clinical samples cannot be reproduced in the human colon cell lines HT-29, CaCo-2 and HCT-116. Possibly, this is due to the absence of a functional micro-environment in the culture and/or by the optimal growth-conditions for the tumor cells *in vitro*. Performing these assays under hypoxic culture conditions might more closely mimic the growth conditions of the colon tumor cells *in vivo* although the addition of TNF-α to the cultures as a stress-trigger had no effect (data not shown).

Alternatively, short-term cultures of fresh samples of colon adenoma and carcinoma may be considered. As these samples contain all the cellular components of real-life tumors, they may be more responsive to the treatment conditions. Hypoxic culture conditions may additionally be applied to mimic *in vitro* the *in vivo* tumor situation as accurately as possible.

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# Part IV

# Discussion and perspectives

# Discussion and perspectives

## The expression of VEGF family members in colon cancer: from Pandora's box to a holistic view

The VEGF family members have been studied many times by many means in many settings on many levels and in many types of specimens. More specific, these "many's" stand for the fact that different research groups have studied VEGFs by a range of different methods (RT-qPCR, PCR, northern blot, western blot, ELISA, immunohistochemistry,…) in different combinations (individual VEGFs, by pairs, by three or by four) at different levels (mRNA or protein) and in different specimens (blood, serum, plasma, cell lines or tissue). We believe that all these 'many's' and 'different's' contribute to the contradictory results that are yielded when performing a literature search for the expression of VEGF family members in colon cancer. Even when considering one specific methodological setting, namely tissue mRNA levels by RT-qPCR, still contradictory reports on the expression levels of VEGFs in colo(n)(rectal) cancer persist [1-6]. Yet, we identified two possible confounding factors in those studies.

First of all, these studies used tissue samples obtained by two different clinical procedures, either resection or biopsy [1-6]. By analyzing the difference between both sample types, we may have opened Pandora's box since we revealed that this "small" difference holds major implications toward the reliability and capacity of VEGF family members as tissue markers for colon cancer. All VEGF family members showed significantly higher expression levels in healthy colon samples obtained by resection as compared to healthy colon biopsy samples. Because VEGFs are inducible by hypoxia [7, 8] and because also the expression levels of hypoxia inducible *COX2* and the hypoxia markers *GLUT-1* and *CAIX* were significantly increased in healthy resection samples, we presume that these expression increments are due to hypoxic stress. In addition, similar expression levels of the hypoxia insensitive *5-LOX* were observed in both sample types, which corroborates our premise. The colon tissue may be prone to hypoxia during surgical resection which takes 30 to 90 minutes. Throughout this time, part of the colon is clamped off and thus this tissue is cut off from the blood circulation and oxygen delivery. Therefore, it is our assumption that hypoxic stress is intrinsic to samples obtained by surgical resection. The consequences for mRNA expression analysis of resection samples were rather dramatic because the hypoxia-induced expression in healthy resection samples, abolished the differential

expression of *VEGF-B* and *VEGF-C* in matched carcinoma resection samples and created a surgery-induced underexpression of *VEGF-D*. As a consequence, based on resection samples, VEGF-B and VEGF-C provided low accuracy and potential as colon cancer mRNA markers whereas underexpression of *VEGF-D* emerged as a strong and potent marker. These observations are however in absolute contrast with those in biopsy samples where *VEGF-B* and *VEGF-C* emerged as potential and carcinoma stage-specific mRNA markers and a differential expression of *VEGF-D* was completely absent. Such a dramatic effect of the sampling procedure on the expression levels of VEGF family members may very well contribute to the contradictory results in literature. In addition, our data indicate that biopsy samples, which require only minutes to collect, provide a more accurate report on the expression levels of hypoxia-sensitive genes like the VEGF family members.

The use of biopsies implies an additional consideration, namely that a single tumor may display several expression signatures, dependent on the location within the tumor. For instance, the invasive edge of a tumor has different needs than a necrotic region in the middle of the tumor, which may translate in a differential expression pattern in both regions. Since tumor biopsies cover only a small part of the tumor, regional differences within the tumor may cause variable expression levels between biopsy samples from a single tumor. It would therefore be interesting to analyze several (characterized) areas from the same tumor but the sampling of multiple biopsies from a single tumor is not something to be taken for granted as it increases the risk of bleeding for the patient drastically. Although no explicit analysis was performed to compare several biopsy samples from the same carcinoma, two independent experiments – each analyzing a combination of 2 biopsies – were performed on a subset of the carcinoma samples (n=23), thus providing datasets on different biopsies from these carcinomas. When comparing the results of both experiments, the main conclusions are identical. Moreover, despite minor methodological differences between both experiments, 87% of these carcinomas showed a similar profile (no or 1 VEGF *versus* co-expression) in both experiments. This indicates at least that the biopsies (n=4) received as a single carcinoma sample, were obtained in regions with rather homogenous expression of VEGFs.

A second possible confounding factor was found in the fact that most expression studies use colorectal samples and thus colon as well as rectal samples [2-6]. However, rectal cancer patients near always receive neo-adjuvant therapy prior to surgery. Therefore, the inclusion of rectal

samples may constitute a confounding factor. As radiation and chemotherapy have been shown to induce hypoxia and upregulation of VEGF-A in experimental as well as clinical samples [9-12], it is not unthinkable that neo-adjuvant therapy upregulates the expression of certain VEGF family members in rectal cancer samples. Further research, involving a comparative expression analysis – as we performed for resection and biopsy samples – is indicated to compare the expression levels of VEGF family members in healthy rectal and rectal carcinoma samples before and after neo-adjuvant therapy. Such an analysis may validate our hypothesis that also the inclusion of rectal samples constitutes a confounding factor.

By exclusion from our analyses samples obtained by resection as well as samples of rectal origin, we avoided interference of these confounding factors. Next, we addressed the limitations of previously published studies on the expression of VEGF family members in colon cancer by a systematic and comprehensive analysis of the mRNA expression levels of all individual human VEGFs in a single experimental setup. To provide an insight in the expression of these genes upon progression from healthy colon to a metastasized disease, also colon adenoma and liver metastasis samples were included.

However, the inclusion of liver metastasis samples, presented a novel complexity; we needed to consider if liver metastases had to be compared with healthy liver – the tissue where they were found – or with healthy colon – the tissue they originate from. The mRNA expression levels of the colon marker TTF3 and the liver markers LXRalpha and APoE in a subset of healthy colon, healthy liver and liver metastasis samples, taught us that liver metastasis samples retained the characteristics of their tissue of origin and gained characteristics of their new environment. Indeed, *TTF3* levels in liver metastasis samples were similar to those in healthy colon and *LXRalpha* and *ApoE* levels resembled those in healthy liver. Probably, this additional liver signature reflects the presence of residential liver cells in the metastasis samples. The contributions of different cell types in the liver metastasis samples to the expression signature could be verified in detail by isolation of specific cell subsets with a laser-dissection microscope. Yet, of most relevance to this thesis is the observation that the ectopic origin of the liver metastases did not affect the conclusions from our comparative VEGF gene expression analysis. In fact, similar (*VEGF-B)* or even higher (*VEGF-A*, *-C*, *-D* and *PlGF*) expression increments were obtained when liver metastasis samples were compared with healthy colon instead of healthy liver. This indicates that for metastasis samples the expression increments may be even

more pronounced than indicated on the basis of using healthy liver samples as calibrator. Nevertheless, we have chosen to follow a conservative approach based on a comparison with healthy liver samples because it is far less obvious in the clinical practice to acquire at the same time healthy colon samples and liver metastasis samples from one individual patient.

The normalization of liver metastasis samples was done with only SDHA, due to instability of TBP as reference gene in liver metastasis samples. Although we realize that at least two reference genes are compulsory for normalization of RT-qPCR analyses, we could not repeat the entire experiment with two (new) stable reference genes due to technical issues. In addition, SDHA was found to be the most stable reference gene out of ten for liver metastasis samples. After careful deliberation, these considerations led to the decision to use only SDHA as a reference gene for normalization of liver metastasis samples. It would be ideally to repeat the entire experiment with at least two reference genes, stable for all the samples analyzed.

Finally, our comprehensive and systematic analysis of the mRNA expression of VEGF family members revealed the complex nature of angiogenic gene expression already at the stage of adenoma and its further deviation towards co-expression of multiple angiogenic genes upon progression to carcinoma and liver metastasis. A clear stage-associated progression was apparent evolving from *VEGF-A* and/or *PlGF* overexpression in adenoma samples toward the additional overexpression of *VEGF-B* and/or *VEGF-C* in carcinoma as well as metastasis samples. Thus, we identified *VEGF-B* and *VEGF-C* as angiogenic genes which are upregulated only in carcinoma and metastasis stages. Individual patient samples revealed correspondingly discriminating signatures with none up to two *VEGFs* overexpressed in adenoma samples and three up to four *VEGFs* in carcinoma and metastasis samples. In addition, we found a striking conservation between colon carcinoma and liver metastasis samples of the *VEGF* expression signatures, indicating that the *VEGF* expression signatures of colon carcinoma are conserved when metastasizing to other body tissues. In order to further corroborate the apparent conservation of *VEGF* expression signatures between primary colon carcinoma and its secondary metastasis, it is indicated to compare the expression levels in liver metastasis samples and primary colon carcinoma samples from the same patient. Regretfully, for practical reasons it was not possible to perform such a matched analysis in the course of the PhD. In addition, it may be interesting to perform similar analyses on colon cancer metastases from other body sites than the liver in order to verify if this conclusion holds true for metastases in other organs.

Ultimately, mRNA-expression is one thing, protein expression is something else. Transcription of DNA to mRNA does not necessarily lead to the translation to a functional protein. Nevertheless, mRNA levels may correlate with protein levels and preliminary data on samples from mouse tumor models have shown similar mRNA and protein expression levels at least for VEGF-A and COX2. Attempts to detect the various VEGF members on human colon carcinoma tissue slides by immunohistochemistry have however failed due to major background staining. The secreted nature of the angiogenic factors was most likely at the basis of the background staining. Alternative methods to analyze the protein levels were impeded by the lack of an available ELISA-test for VEGF-B and the limited sample size of human biopsies. These limitations have obliged us to remain with the quantification of mRNA expression levels, although a parallel mRNA-protein profiling is indicated to further corroborate the results discussed in this study.

**VEGFs as diagnostic markers for colon cancer**

Worldwide, colon cancer is the second leading cause of cancer-related mortality and accounts for over 1,2 million new cases on a yearly basis. Colon carcinoma evolves from a premalignant precursor stage, the so-called polyps or adenoma, through a multistep process, which involves sequential and cumulative genetic and epigenetic alterations. Early detection, an essential prerequisite to increase patient survival, is difficult by clinical presentation because of the asymptomatic nature of colon carcinoma and its adenoma precursor stage. At present, screening of the population at risk (age 50 and over) is hampered by the lack of patient-friendly, sensitive and affordable detection methods. Endoscopic examination of the colon by colonoscopy is still the gold standard for the detection of colon adenoma and carcinoma but its broad application is strongly hampered by the invasiveness of the procedure, thus rendering the procedure unpopular and unfit for large-scale screening of the population at risk. Non-invasive screening methods for the early detection of colon adenoma and carcinoma are therefore urgently needed.

Because the VEGF family members are readily secreted upon gene induction, we verified to what extent the increment in PlGF and VEGF-A mRNA levels observed in colon carcinoma and colon adenoma biopsies translates into increased levels in the blood, thus effectively verifying the potential of PlGF and/or VEGF-A blood levels to serve as biomarker for the presence of colon carcinoma and colon adenoma. The validation of PlGF and/or VEGF-A as a serum biomarker for colon carcinoma and adenoma would, if successful, offer a much needed non-invasive and cost-

effective screening method for the early detection of colon cancer. In addition, also VEGF-C serum levels were determined to verify if they could serve as a blood marker, specific for the carcinoma stage. Despite previous studies reporting increased levels of serum VEGF-A and VEGF-C in colon cancer patients [13-16], we found for neither of them differential serum levels in healthy individuals compared to patients with colon adenoma or carcinoma. However, there is considerable controversy about the ideal type of blood sample – serum or plasma – for measurement of VEGF-A and VEGF-C since both factors are also released by platelets upon blood clotting, which is applied to obtain serum [17-19]. Platelet-derived VEGF-A and/or VEGF-C in serum may thus increase background values and hence mask tumor-derived increments. There is therefore an urgent need for a systematic analysis of VEGF-A and VEGF-C levels in different blood sample types. A potential experiment to address this query would be an ELISA performed on serum and plasma from the same patients in combination with platelet counts to allow normalization of differential platelet numbers between the samples, as has been suggested by George and colleagues [20]. Such a systematic analysis may clearly determine the differences between serum and plasma levels, taking into account a potential contribution of platelets. A simultaneous analysis of the expression of the angiogenic genes in adenoma or carcinoma tissue, may than allow a further comparison between tissue and blood levels and determine whether plasma or serum levels provide the best reflection of VEGF-A and VEGF-C levels in the diseased tissue. Such approach may resolve the current discrepancies and lead to the establishment of a standard method for the measurement of VEGF-A and VEGF-C in blood.

On the other hand, serum levels of PlGF were significantly higher in samples from patients with colon carcinoma than in samples from healthy individuals. Increased serum levels of PlGF have been shown before in colorectal cancer [21, 22] but no reports are available in literature for PlGF serum levels in colon adenoma. We did not observe significant higher levels of PlGF in serum of patients with colon adenoma, although a tendency of an increment was apparent. Thus, the serum levels of PlGF may correlate with the mRNA expression profile of PlGF, at least at the stage of colon carcinoma. In order to validate these observations, an extended population study is required in which PlGF blood levels are determined and correlated with the results of endoscopic examination by colonoscopy for the presence of colon adenoma or carcinoma.

**VEGFs in combination with eicosanoid enzymes as markers for colon cancer**

Research preceding this thesis has demonstrated in mouse tumor models that the contribution of COX2-derived prostaglandins to the growth and malignancy of tumors is considerably more complex than anticipated [23]. Based on different mouse tumor models, it appears that besides the well-known COX2-dependent expression of *VEGF-A* [24, 25], also a COX2-independent deregulated *VEGF-A* expression may exist. The occurrence of COX2-independent expression in highly aggressive tumor models indicated that this deregulated expression of *VEGF-A* may be associated with a high degree of malignancy [26]. Therefore, the original aim of the current study was to translate these observations in mouse tumor models to human colon cancer. The hypothesis that COX2-independent expression of *VEGF-A* may be associated with malignancy in human colon cancer was however rapidly overthrown by our data, showing high levels of *COX2* in most of the colon carcinoma samples (92%), thus demonstrating that a COX2-indepent expression of *VEGF-A* does not occur in human colon cancer. On the other hand, the systematic analysis of mRNA expression levels of COX2, 5-LOX and VEGF family members in colon adenoma and carcinoma revealed other interesting findings. Firstly, *5-LOX* was the predominant eicosanoid enzyme in colon adenoma samples, whereas in carcinoma samples it was mainly *COX2*. This may indicate that both enzymes are involved in defined steps of tumorigenesis and malignancy. Secondly, adenoma samples with upregulation of both *5-LOX* and *COX2* were at greater risk of malignant transformation into carcinoma (Figure I). Expansion of the population study to a larger cohort size clearly is indicated to further corroborate these findings and to determine the frequency of false negatives. At this point, we cannot exclude that the observed association with malignancy is to be attributed to COX2 alone, rather than to the dual expression of *COX2* and *5-LOX*. COX2 in adenoma samples has been described in association with typical risk factors of malignant transformation of adenoma [27-29]. Our study revealed a correlation between *COX2* expression and the grade of dysplasia in adenoma samples, which was not found for 5-LOX. In addition, although colon carcinoma cells became more invasive *in vitro* when either $PGE_2$ or $LTD_4$ were added to the culture medium, there was no additional increase in invasiveness when both agonists were added. On the other hand, elimination of either COX2- or 5-LOX-derived eicosanoids *in vitro* abolished the observed increase in invasion by respectively $LTD_4$ or $PGE_2$, indicating that both are required for increased invasiveness. Short-term cultures of freshly isolated colon adenoma tissue may allow to determine if either COX2 or the

combination of both eicosanoid enzymes causes a higher risk of malignant transformation. Finally, we found that the overexpression of eicosanoid enzymes was associated with the cumulative co-expression of VEGF family members in both adenoma and carcinoma samples (Figure I) and that the expression of *COX2* was associated with the expression of *VEGF-A*, *VEGF-C* and *PlGF*.

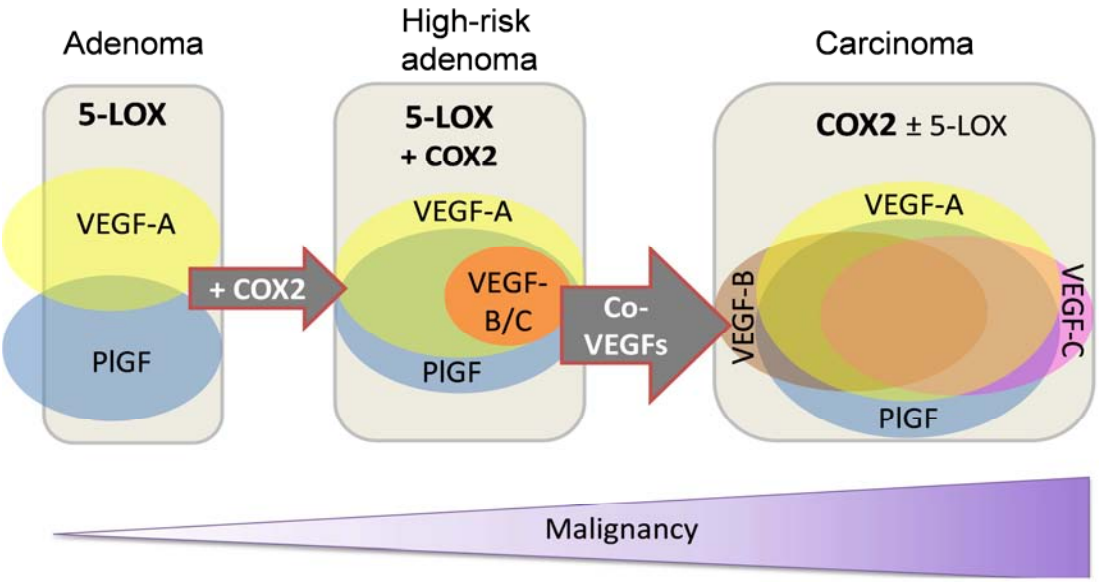


**Figure I – Schematic summary of the intertwined inflammatory and angiogenic gene expression in colon adenoma and upon progression to carcinoma.** Adenoma samples show a pronounced signature with predominant expression of 5-LOX as well as VEGF-A and/or PlGF. Adenoma with COX2 expression in addition to 5-LOX have an increased risk for malignant transformation to carcinoma. The overall signature of carcinoma samples is a predominant expression of COX2 with the co-expression of three to four VEGF family members.

Nevertheless, we failed to reproduce *in vitro* any association between eicosanoids and VEGF family members. Future attempts to reproduce these correlations *in vitro* may involve the implementation of hypoxic conditions to the cell cultures to better mimic the *in vivo*. The lack of responsiveness of colon carcinoma cell lines to the addition of eicosanoids and their inhibitor, may indicate that the observed signatures in human carcinoma samples are largely attributable to the micro-environment of the tumor. Nevertheless, the tumor cells are likely steering the expression signatures as indicated by the conservation of the signatures between colon carcinoma and liver metastasis samples. Because established cell lines are derived from a single clone of cells and lack therefore the diversity of cancerous as well as environmental cells, they may in fact be incompetent to reproduce the observed signatures. Therefore, the use of short-term cultures of

fresh colon adenoma and carcinoma samples may be more successful than using established cell lines because these clinical samples contain all the cellular components of real-life tumors.

**Considerations for therapy**

Angiogenesis and lymphangiogenesis are important biological responses that crucially contribute to the outgrowth of solid tumors and metastases. Targeting these processes and their mediators as a consequence holds important therapeutic promises. The VEGF family members are crucial angiogenic factors involved in every mechanism of tumor vascularization. However, drugs interfering with VEGF-A or its receptors only show modest effects in most cancers and resistance tends to develop after a transitory period of clinical benefit. Angiogenic escape responsible for this limited response to VEGF-A directed angiogenic therapy is among others stimulated by the compensatory expression of VEGF-related angiogenic factors. In line herewith, increased expression levels of VEGF-A, -B, -C and PlGF have been shown in colorectal cell lines after chronic exposure to bevacizumab [30]. Our comprehensive and systematic analysis of the VEGF family members in colon cancer revealed indeed the co-expression of three to four *VEGFs* in colon carcinoma and liver metastasis samples. This excessive expression of multiple angiogenic genes may contribute to the observed resistance toward VEGF-A targeting therapy. The assessment of VEGF expression profiles in samples before and after treatment with anti-angiogenic therapy and in samples at apparent therapy-resistance is indicated to determine if the treatment stimulates a further upregulation of VEGF family members, which would indicate that they are effectively contributing to therapy-resistance. Considering the observed association between eicosanoids and co-expression of VEGF-family members, patients may benefit from a combination therapy targeting angiogenic VEGF signaling as well as eicosanoids to additionally suppress the co-expression of multiple VEGFs.

**Tumor size and hypoxia, future perspectives on the impact of angiogenic gene signatures**

Colon cancer is staged according to the TNM classification whereby T represents the mural depth of the tumor. Because the size of a colon tumor is not necessarily related to the stage of the cancer, clinicopathological data concerning the size of carcinomas were incomplete and mainly TNM scores were documented in the thesis. However, the size of a tumor may possibly influence its hypoxic conditions and thus the expression levels of hypoxia-sensitive genes as COX2 or VEGF family members. The determination of expression levels of hypoxia markers in addition to

the gene-set analyzed in this thesis, may elucidate whether expression levels of VEGFs and/or COX2 are correlated with those of hypoxia markers and thus with the level of hypoxia within a tumor. Supplementary, it may be interesting to perform this experiment on carcinoma samples with characterized size and vascularity to analyze an eventual association with clinicopathological features.

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# Part V

# Addendum

# Curriculum Vitae

## Personal Information


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## Educational background

2007-2013: **Ph.D. in Science: Biotechnology**

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2002-2006: **Master in Biomedical Sciences**

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1996-2002: **Latin-Sciences**

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2010-2011: Advanced academic English: Writing Skills Life Sciences and Medicine
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2006-2007: Basic course in Laboratory Animal Science, Cat B
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## List of publications

Pringels S, Van Damme N, De Craene B, Pattyn P, Ceelen W, Peeters M and Grooten J. (2012) Clinical procedure for colon carcinoma tissue sampling directly impacts the cancer marker-capacity of VEGF family members. BMC Cancer 12:515.

Pringels S, Van Damme N, Geboes K, De Craene B, Troisi R, de Hemptinne B, Peeters M and Grooten J. Stage-specific cumulative expression of VEGF family members during colon cancer progression. [submitted]

Pringels S, Van Damme N, Geboes K, De Craene B, Peeters M and Grooten J. Intertwined inflammatory and angiogenic shift during malignant transformation of colon adenoma into colon carcinoma. [in preparation]

## Presentations

### Oral presentations

* BACR meeting, Brussels, 31 January 2009, "Correlated expression analysis of VEGF family members and eicosanoids in human colon polyps and carcinomas and liver metastases"

* WOG meeting, Leuven, 27 March 2009, "Correlated expression analysis of VEGF family members and eicosanoids in human colon polyps and carcinomas and liver metastases"

* DMBR Workshop: The Tumor Host Interaction Program, Ghent, 8 June 2009, "Correlated expression analysis of VEGF family members and lipid inflammatory mediators in human colon polyps and carcinomas and liver metastases"

* 5th ICTM meeting: Progression, Therapy and Prevention, Versailles, 20-24 October 2009, "Correlated expression analysis of VEGF family members and lipid inflammatory mediators in human colon polyps and carcinomas and liver metastases"

### Poster presentations

* EACR-21, Oslo, 26-29 June 2010, "Correlated expression analysis of VEGF family members and lipid inflammatory mediators in human colon polyps and carcinomas and liver metastases"

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*Supervision of Magdalena Byszewska, Erasmus Exchange Program

# Dankwoord

# Dankwoord

Zo zijn we dan (uit)eindelijk aangekomen bij pagina 175 en de laatste (nu nog) lege pagina's die beschreven moeten worden. Deze keer geen wetenschappelijke cijfers of overdenkingen maar wel een dankwoord om allen die op één of andere manier hebben bijgedragen tot deze thesis te bedanken en dat zijn vele mensen…

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